A study of Immune responses to *L. mexicana* Antigens and Immunogenicity of *L. donovani Centrin-3*

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Abstract

Leishmaniasis is a parasitic protozoal disease affecting humans and animals with *phlebotomine* sand flies as intermediate vectors. The parasite infects phagocytic cells of the mammalian host where they transforme from the flagellated promastigote to non-flagellated amastigote phase. There is no effective vaccine in use against this parasite and production relies on finding potent immunogenic antigens with a Th1 bias and long lasting memory response. In this study the immunogenicity of *L. mexicana* Soluble *Leishmania* Antigens (SLA) prepared by two different methods (SLA1&2) was investigated by immunisation of Balb/c mice and challenge with live *L. mexicana* and an *in vitro* immunological analysis. Immunisation of Balb/c mice with SLA mixed with IFA adjuvant significantly protected against challenge with live *L. mexicana* parasites. The SLA2 was also further fractionated into six sub fractions by fast protein liquid chromatography (FPLC) using Mono Q columns and the immunogenicity of each fraction was analysed either by ability to stimulate CTL activity against dendritic cells (DCs) target cells loaded with SLA2 and SLA2 fractions or by tritiated thymidine uptake proliferation assay. Immunisation of Balb/c mice with whole SLA as well as the SLA2 fractions induced a significant CTL activity, but responses were higher for the whole SLA. Splenocytes stimulated *in vitro* for 7 and 14 days with SLA2 and SLA2 fractions induced significant proliferation responses which was increased when splenocytes were stimulated with DCs loaded with these antigens.

Leishmania parasites require a number of immune-evasion mechanisms to resist phagolysosome fusion and prevent activation of more-potent acquired immune

responses. Down regulation of MHC class I and II expression on infected phagocytic cells may be one of the immune evasion strategies used by the *Leishmania* parasite. In this study the effect of *L. mexicana* infection on the expression of surface molecules was investigated in DCs. Unlike treatment with autoclaved parasite, infection of DCs with live *L. mexicana* parasite down regulated the expression of MHC class I, class II, CD11c, CD80 and CD40. Also, *in vitro* treatment of DCs with fungizone as early as 1 hour after the initiation of infection with *L. mexicana* restored their MHC class I expression, as determined by antibody staining and flow cytometry analysis. Interestingly treatment of *L. mexicana* infected DCs with fungizone also restored their susceptibility to CTL activity.

As part of searching for new *Leishmania* antigens of a potential vaccine application, the immunogenicity of *L. donovani centrin-3* (*Ldcen-3*) was investigated in a Balb/c model. The immunogenicity of *Ldcen-3* has not previously been investigated. *Ldcen-3* is a calcium binding protein that has been shown to be involved in duplication and segregation of the centrosome in higher and lower eukaryotes. The *Ldcen-3* gene was cloned in various vectors and coated on gold particles for gene gun immunisation. Significant protection was induced by immunisation with 1μg DNA of pcDNA3.1-*Ldcen-3* or pCRT7/CT-TOPO-*Ldcen-3* constructs. Protection against challenge with live parasite was vector dependent where better protection was induced by pCR T7/CT-TOPO-*Ldcen-3*. Splenocytes from Balb/c mice immunised with pcDNA3.1-*Ldcen-3* or pCRT7/CT-TOPO-*Ldcen-3* has a potent CTL response against DC targets loaded with SLA or tumour cells transfected with *Ldcen-3* plasmid construct.

Collectively, results presented in this study suggest that the whole SLA was more immunogenic than any SLA fractions produced by fast protein liquid chromatography. Results also suggest that *L. mexicana* could use down regulation of MHC I as a possible mechanism to evade killing by CTL and susceptibility to CTL could be restored by treatment of infected cells with fungizone. These findings also suggest the potential benefit of combination therapy in controlling *Leishmania* infection. This study has also investigated for the first time the immunogenicity of *Ldcen-3* gene which was shown to be highly immunogenic via protection against challenge with live parasite and induction of CTL in immunised mice.

Abbreviations

ACL	American cutaneous Leishmaniasis
ACP	Amastigote Cysteine Proteinase
Ad5IL-12	Administration of an adenovirus expressing IL-12
AdjuPrimeTM	carbohydrate polymer AdjuPrimeTM
ADV	Adenovirus
Ag	Antigens
ALA	Autoclaved *Leishmania* Antigen
ALM	Autoclaved *L. major*
Alum-ALM	Alum autoclaved *Leishmania major*
APC	Antigen Presenting Cells
BCA	Bicinchoninic Acid
BCG	Bacillus Calmette-Guerin
BM-DC	Bone marrow- Dendritic Cells
BSA	Bovine Serum Albumin
CFA	Complete Freund's Adjuvant
CL	Cutaneous Leishmaniasis
CMV	cytomegalovirus
CP	Cysteine Proteinase
CP	*Corynebacterium parvum* (adjuvant)
CPi	cysteine protease inhibitors
CT	cholera toxin
CTL	Cytotoxic T- Lymphocyte
CVL	canine visceral Leishmaniasis
CWS	cell wall skeleton
DC	Dendritic Cells
DCL	Diffuse Cutaneous Leishmaniasis
DDT	DichloroDiphenylTrichloroethane
DHFR-TS	Dihydrofolate Reductase Thymidylate Synthase gene
dLNs	draining lymph nodes
dNTP	Deoxy nucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay

EU	European Union
FCS	Foetal Calf Serum
FML	Fucose-mannose ligand
FPLC	Fast performance liquid chromatography
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
gp63	glycoprotein 63 KD
gp46	glycoprotein 46 KD
GPI	glycosylphosphatidylinositol
GPIL	glycoinositol phospholipids
HASPB1	Hydrophilic Acylated Surface Protein B1
HBcAg	Hepatitis B core Antigen
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
I.D.	Intradermal
IFA	Incomplete Freund's adjuvant
IFAT	Indirect fluorescent Antibody Test
IFN-γ	Interferon-γ
Ig	Immunoglobulins
I.M.	Intramuscular
I.N.	Intranasal
iNOS	inducible Nitric oxide synthase
I.P.	Intraperitoneal
KCs	Kupffer cells
KMP-11	*L. donovani* Kinetoplastid Membrane Protein 11
KOAc	potassium acetate
LACK	*Leishmania* homolog of receptors for Activated C Kinase
Lag	*L. donovani* promastigote antigens
LaAg	*L.* amazonensis promastigote antigens
L110f	*Leishmania* poly-protein
LCL	Localized Cutaneous Leishmaniasis
LCs	Langerhans cells
Ldcen-1	*Leishmania donovani centrin-1*
Ldcen-3	*Leishmania donovani centrin-3*
LeIF	*Leishmania* elongation initiation factor
Leish-111f	*Leishmania*-derived recombinant polyprotein
LiESAp	*L. infantum* excreted supernatant antigens promastigotes

L110f	Formulated *Leishmania* poly-protein
LmSEAgs	*L. major* exo-antigens
LmSTI1	*L. major* stress inducible protein 1
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LRP	*L. major* ribosomal protein
LST	*Leishmanin* Skin Test
MAC	membrane attack complex
Man5-DPPE	liposomes coated with dipalmitoylphosphatidylethanolamine
MBP	Mannan-binding protein
MØ	Macrophages
MCL	Mucocutaneous Leishmaniasis
MHC	Major Histocompatibility Complex
mDsRed	monomeric red fluorescent protein
MML	*Leishmania* poly protein
MPL	Monophosphoryl lipid
MPL-A	monophosphoryl lipid A
MPL-SE	monophosphoryl lipid A in stable emulsion
MTOC	microtubule organising center
MyD88	Myeloid differentiation primary response gene (88)
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NH36	Nucleoside Hydrolase 36
NK	Natural Killer cells
NKT	Natural Killer T cells
NO	Nitric Oxide
NWDCL	New World Diffuse Cutaneous Leishmaniasis
O_2^-	Superoxide anion
OX40L	OX40 ligand
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PCR	Polymerase Chain Reaction
PKDL	Post-kala-azar dermal Leishmaniasis
PMNs	Polymorphonuclear leukocytes
PQ	Recombinant protein formed by the genetic fusion of four cytoplasmic proteins
PPGs	proteophosphoglycans

PSA	promastigote surface antigen
PVP	polyvinylpyrollidone
RES	Reticuloendothelial system
S.C.	Subcutaneous
ScLL	Synadenium carinatum latex lectin
SLA	Soluble *Leishmania* Antigen
SPi	serine protease inhibitors
TAg	Total Antigen of *Leishmania chagasi*
TBS-T	Tris Buffered Saline and Tween 20
TCR	T-cell Receptor
TDM	trehalose dicorynomycolate
TEMED	Tetramethylethylenediamine
TGF-β	Transforming Growth Factor-β
Th1	T helper1
Th2	T helper2
TLR	Toll-Like receptors
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
TRYP	Tryparedoxin peroxidase
TSA	Thiol-Specific antioxidant
VL	Visceral Leishmaniasis
VLP	virus-like particle influenza
WHO	World Health Organisation

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1 Introduction

1.1 *Leishmania*

The causative agent of Leishmaniasis, a disease causing suffering similar to malaria, was first described by William Leishman and Charles Donovan in 1903 (Jacobson, 2003). The *Leishmania* species are vector-borne, protozoans which are responsible for a group of diseases referred to as Leishmaniasis (Wheat *et al*., 2008). The impact of *Leishmania* is universal, as human Leishmaniasis is endemic in 88 countries worldwide and has an approximate incidence of 20 million cases with about 2 million new cases occurring annually (Awasthi *et al*., 2004; Rodriguez-Cortes *et al*., 2007). Annually, Leishmaniasis is responsible for 60,000-70,000 deaths worldwide and an estimated 10% of the world's population is at risk of infection and disease (Reithinger *et al*., 2007; Rodriguez-Cortes *et al*., 2007). Leishmaniasis is also considered as an emerging disease in Europe, the risk of spreading of Leishmaniasis among EU States has recently been evaluated for a short 2 to 3 and long-term 15 to 20 years. The main threat comes from the spread of two species of the parasites that are endemic in the EU, *L. tropica* and *L. infantum*, which cause cutaneous and visceral Leishmaniasis, respectively (Ready, 2010). Having been identified as a category one disease by the World Health Organisation (WHO), Leishmaniasis is considered as an emerging and uncontrolled disease and with the advent of HIV, *Leishmania* co-infection is a rising cause for concern in South Europe (Piscopo & Mallia 2007; Reithinger *et al*., 2007). The *Leishmania* organism is an obligatory intracellular parasite of macrophages and other phagocytic cells which are spread by the bite of sandflies (Gomes *et al*., 2007; Rafati *et al*., 2011). However, the disease is wide spread and exists mainly in the

tropical and subtropical regions of the world (McConville *et al*,. 2007). *Leishmania* parasites are dimorphic, with two morphologically distinct stages that can be identified dependent on hosts and developmental stages (Fig 1.1). The first form is termed the promastigote stage where it is elongated in shape and consists of long flagella. Parasites are found in a promastigote form in the sand fly vector (Alvar *et al*., 1992; Magill, 1995; Wilson, 2010). The promastigote form can be further divided into procyclic promastigotes, which are found in the gut of the sand fly and which actively, multiply and metacyclic promastigotes, which are found intracellular in the anterior gut and mouth region and do not multiply. The other form is called the amastigote, which is found in the host. Unlike promastigotes, this form lacks the flagella and are round or oval in shape (Olivier *et al*., 2005). Apart from the difference in the location and morphological form, there is also a difference in the presence and amount of surface molecules in the different developmental stages of the parasites. For example, the metacyclic promastigotes have a thicker glycocalyx when compared to procyclic promastigotes, whereas the glycocalyx is absent in amastigotes (Pimenta *et al.*, 1991). The structure of LPG (*Leishmania* possess a lipophosphoglycan coat over the outside of the *Leishmania* cell) is longer in metacyclic promastigotes compared to procyclic promastigotes and almost not found in amastigotes. The gp63 is more abundant in promastigotes compared to amastigotes (McConville & Blackwell, 1991; Abu-Dayyeh *et al*., 2010).

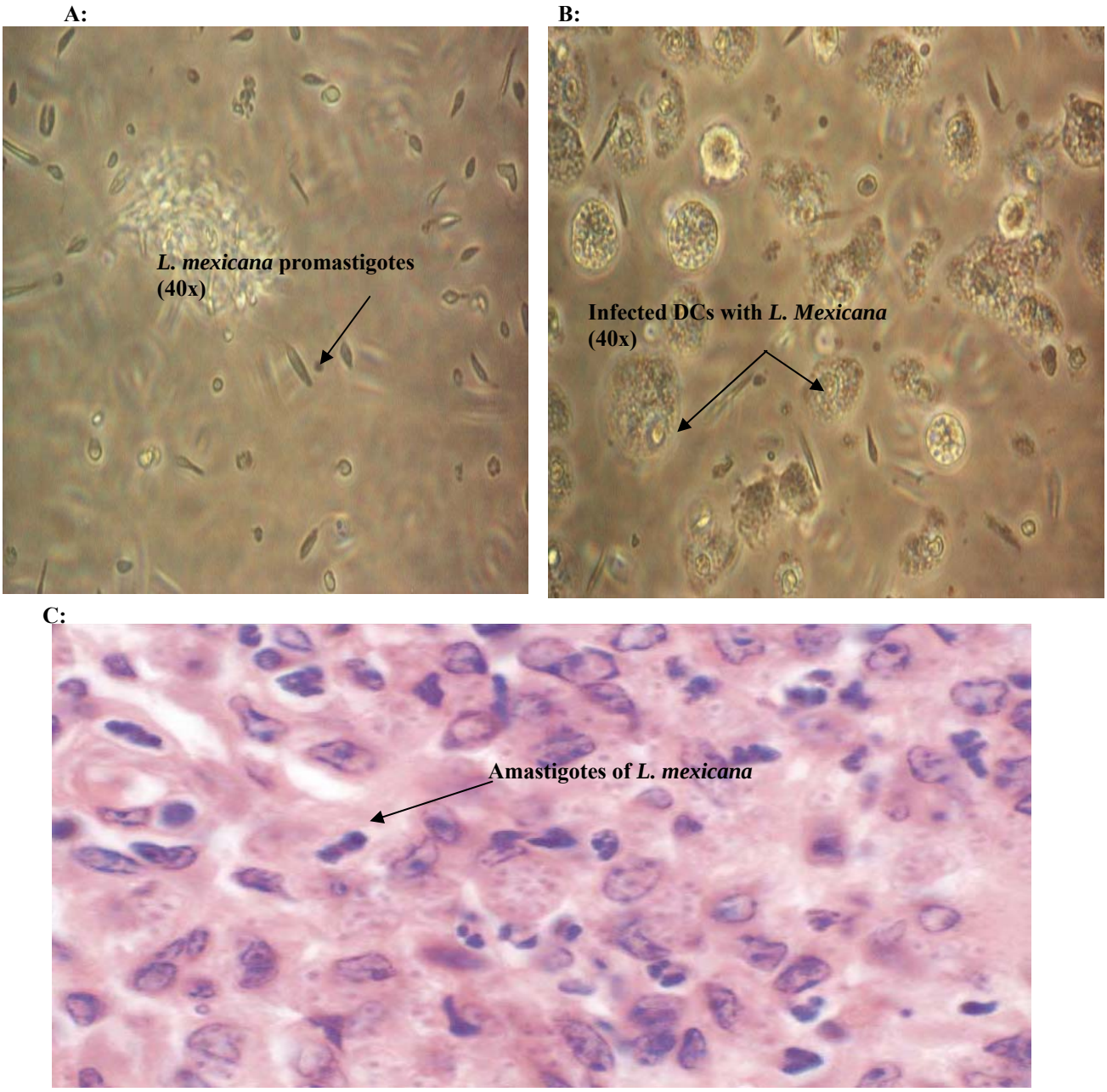


Figure 1.1 A: Cultured promastigotes of *L. mexicana* alone or B: in infected dendritic cells at NTU lab and C: amastigotes *L. mexicana* infected macrophages (Costa *et al*., 2003).

1.2 Classification of *Leishmania* species

Leishmaniasis is divided into four major clinical forms and is caused by parasitic protozoa of the genus *Leishmania*. There are more than 20 species and subspecies that infect humans. The clinical features of the disease depend on the causative species

and can range from simple, self-healing skin sores as found in cutaneous Leishmaniasis, to severe, life-threatening untreated visceral Leishmaniasis caused by *Leishmania donovani*. Classification and taxonomy of the *Leishmania* parasite, along with its geographical distribution is tabulated in (Table 1.2). The exact identification and classification of species of *Leishmania* is important for epidemiology and control of the disease because various species of *Leishmania* cause various clinical types of the disease (Mimori *et al*., 1998; Mishra *et al*., 2009). Leishmaniasis diagnosis can be made on the basis of epidemiological and clinical data but has to be confirmed by laboratory tests to avoid potential misdiagnosis. Because of differences among the *Leishmania* species in levels of virulence and response to chemotherapeutic regimens, correct identification is essential in order to determine the clinical prognosis and prescribe an appropriate species-specific therapeutic regimen (Berman, 1997; Mizbani, *et al.*, 2009).

Taxonomy of *Leishmania* parasite

Kingdom: Protozoa

Subkingdom: Protista

Phylum: Sarcomastigophora

Sub-phylum: Mastigophora

Class: Zoomastigophora

Order: Kinetoplastida

Suborder: Trypanosomatina

Genus: *Leishmania*

Species:

Subgenus	Complex	Species	Main geographic locations	Main clinical manifestation
Old World				
	L. donovani	*L. donovani*	India,subSaharan Africa, China,Pakistan	VL
	L. donovani	*L. infantum*	Mediterranean, Middle East, north and sub-Saharan Africa, Balkans, China	VL
	L. major	*L. major*	Middle East, Africa, India, China	CL ("wet ulcer")
	L. tropica	*L. tropica*	Middle East, India, southern Europe, western Asia	CL ("dry ulcer")
	L. aethiopica	*L. aethiopica*	Ethiopia, Kenya, Yemen	CL
New World				
Subgenera *Leishmania*	*L. donovani*	*L. chagasi*	Latin America	VL
	L. mexicana	*L. venezuelens*	Venezuela	CL
	L. mexicana	*L. mexicana*	Mexico, Central America, Texas, Oklahoma	CL
	L. mexicana	*L. amazonensis*	Amazon basin, Brazil	CL
Subgenera Viannia	*L. braziliensis*	*L. braziliensis*	Latin America	CL and MCL
	L. braziliensis	*L. peruviana*	Peru and Argentina (highlands)	CL
	L. guyanensis	*L. guyanensis*	Northern Amazon basin, Guyanas	CL
	L. guyanensis	*L. panamensis*	Panama, Costa Rica, Columbia	CL

Table 1.2 Classification of *Leishmania* species in new and old world: VL: Visceral Leishmaniasis; CL: Cutaneous Leishmaniasis and MCL: Mucocutaneous Leishmaniasis. *Leishmania* divided in two subgenus: *viannia* and *Leishmania* (Bari & Rahman, 2008).

1.3 Life cycle of *Leishmania*

The method of transmission of *Leishmania* diseases is similar for all *Leishmania* species. When female sandflies of the genus *Phlebotomus* infected with *Leishmania* take a blood meal from the host, the promastigote stages in their gut can penetrate the skin tissue where they are phagocytosed by macrophages (Fig 1.3). Promastigotes resist lysis in the phagolysosome and they transform to amastigote stages where they develop by binary fission (Bogdan & Rollinghoff, 1998; Handman, 2001; Azizi *et al*., 2009). The free amastigotes released from infected macrophages are phagocytosed by other macrophages, bloodstream monocytes or dendritic cells. While the spread of infections by *L. tropica* and *L. major* is limited to the draining lymph node, the *L. infantum* and *L. donovani* move into the whole Reticuloendothelial system (RES), *e.g.,* liver, spleen and bone marrow. The local or general decrease of macrophages may lead to hepatomegaly and/or splenomegaly in visceral Leishmaniasis and lesions in cutaneous infections (Bogdan & Rollinghoff, 1998; Handman, 2001; Okwor *et al*., 2009).

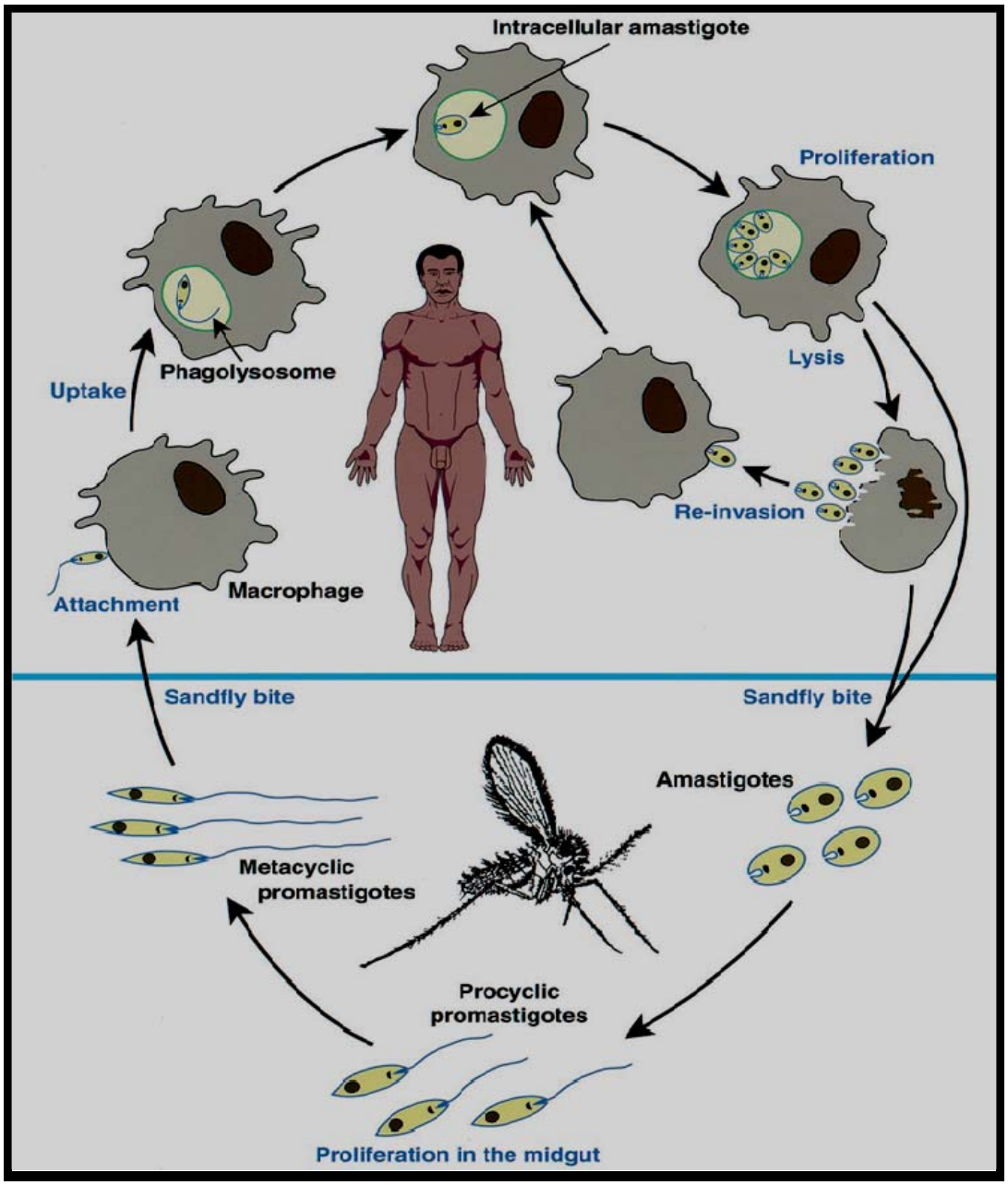


Figure 1.3 Life cycle of *Leishmania*
Female sandflies infected with promastigote stages in their gut transfer the infection to the new host during the feeding process where the promastigotes are phagocytosed by host macrophages and other phagocytic cells. Promastigotes are released in the phagolysosome where they transform to amastigote stages and divide by binary fission (Handman, 2001).

1.4 Leishmaniasis clinical manifestations

The clinical manifestations of human Leishmaniasis depend on the parasite species and on the immune response of the host. The virulence of Leishmaniasis may range

from the more benign cutaneous Leishmaniasis (CL) to visceral Leishmaniasis (VL). In VL the parasite infects and devastates macrophages of the spleen, bone marrow and liver finally leading to organ failure and death (Dumonteil *et al*., 2003; Navarini *et al*., 2009).

1.4.1 Cutaneous Leishmaniasis

CL, mainly caused by *L. mexicana, L. tropica* and *L. major,* is self-healing and causes skin lesions on any part of the body, but mostly on the face, arms and legs (Fig 1.4.1) (Selvapandiyan *et al*., 2006). Cutaneous Leishmaniasis is epidemic in many countries, including Pakistan and Afghanistan (Murray *et al*., 2005; Gazoza., *et al* 2010). The major symptoms of cutaneous Leishmaniasis include the presence of skin ulcers on exposed body regions after a sandfly bite. This type of infection generally heals spontaneously within a few months (3-6) (Schwartz *et al*., 2006).

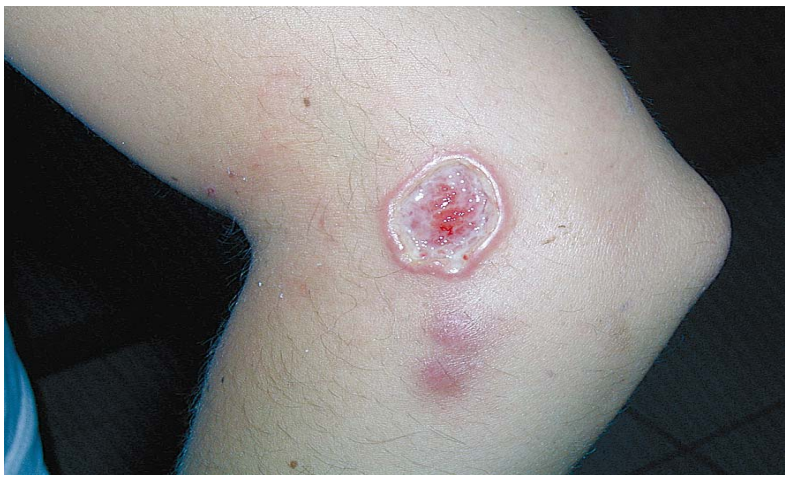


Figure 1.4.1 Cutaneous Leishmaniasis by *L. braziliensis* (Schwartz *et al*., 2006).

1.4.2 Visceral Leishmaniasis

Visceral Leishmaniasis (VL) is caused by many species including *L. donovani* and *L. chagasi.* This disease is also known as 'Kala Azar' and can prove to be fatal if not

treated. More than 90% of visceral cases worldwide arise in India, Sudan, Bangladesh, and Brazil. Its major clinical symptoms include anaemia, fever, cachexia, hepato-splenomegaly and suppression of the cellular immune response (Fig 1.4.2) (Croft, 2008; Gamboa-Leon *et al*., 2006; Subba *et al*., 2008; Sundar *et al*., 2000). However, Post-kala-azar dermal Leishmaniasis (PKDL) a cutaneous manifestation of VL is characterized by skin lesions, nodules or papules, frequently erupting on the face. It often appears 2-7 years after the apparently successful treatment of VL by pentavalent antimony drugs. PKDL is caused by *Leishmania donovani* in India and Sudan, with a few cases reported to be caused by *L. infantum*, *L. chagasi* and historically by either species in China. To date there has been no substantial explanation or treatment for this type of Leishmaniasis (Dey & Singh, 2007; Moore & Lockwood, 2010).

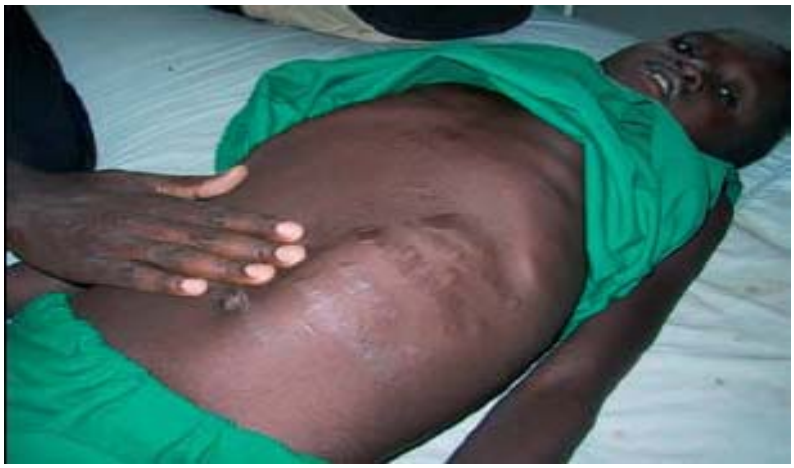


Figure 1.4.2 Visceral Leishmaniasis, (Chappuis is *et al.,* 2007).

1.4.3 Mucocutaneous Leishmaniasis

Mucocutaneous Leishmaniasis (MCL) affects mucous membranes mainly in areas where mucous is attached to skin. This form of Leishmaniasis is also called 'Espundia' (Fig 1.4.3) (Awasthi *et al*., 2004). MCL is a severe and debilitating form

of American cutaneous Leishmaniasis (ACL) caused by infection with *Leishmania braziliensis* (Cabrera *et al*., 1995; Shimabukuro *et al*., 2010).

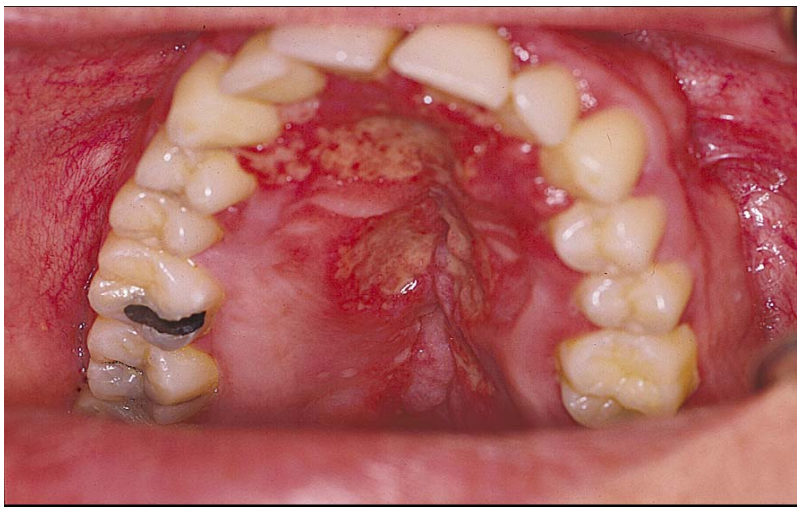


Figure 1.4.3 Mucosal Leishmaniasis, (Schwartz *et al*., 2006).

1.4.4 Diffuse Cutaneous Leishmaniasis

New World DCL (NW-DCL) is a serious sickness caused by parasites of the *Leishmania* subgenus in patients with reduced cell-mediated immunity (anergic) to *Leishmania* parasites. The results of the skin test reaction to *Leishmania* antigens (LST) in these patients are negative (Fig 1.4.4). NW-DCL has been reported in several countries in Central and South America, and is characterized by widely disseminated non-ulcerating skin nodules, papules, and plaques (WHO, 1990). DCL also does not heal spontaneously and is highly resistant to chemotherapy. Presently, there is no effective treatment for NW-DCL and treatment with antimonial drugs or other drugs produces only transitory remissions (Calvopina *et al*., 2006).

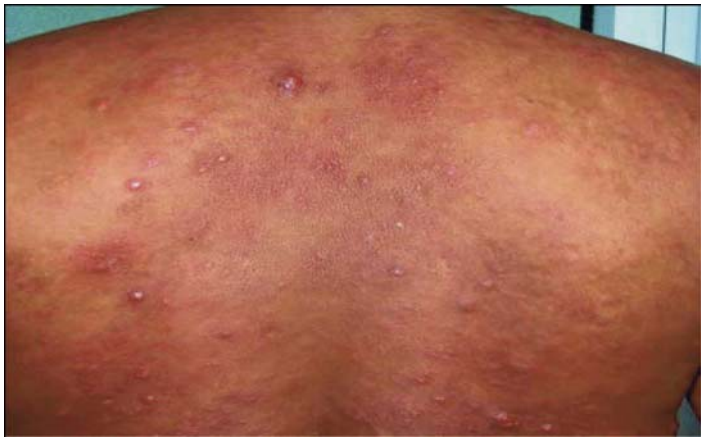


Figure 1.4.4 Diffuse cutaneous Leishmaniasis (Calvopina *et al.*, 2006).

1.5 Control of *Leishmania*

1.5.1 Vector control

Control of Leishmaniasis mainly depends on its epidemiological features. Control methods include elimination of sandflies by environmental and chemical control in the places where carriers are involved and destruction of dogs; which is the major vertebrate host (Claborn, 2010). In India, Nepal and Bangladesh, where chemical control is the only choice of control for visceral Leishmaniasis, most methods to disrupt any vector borne disease are aimed at decreasing man-vector contact. The principle behind environmental control is to manage the environment to make it unsuitable for insects to breed. The DDT spray operation has reduced the sandfly population to very low levels, resulting in interruption of kala-azar transmission and virtual elimination of the disease (Kishore *et al*., 2006).

1.5.2 Chemotherapy of *Leishmania*

The treatment of the *Leishmania* parasite is difficult; it requires time and the application of toxic drugs. Leishmaniasis treatment is also very expensive especially for developing countries and has side effects (Suryawanshi *et al*., 2008). Drugs, like pentavalent antimony, have disadvantages: patients are hospitalised for 3 to 4 weeks for parenteral therapy, which is usually accompanied by side effects such as nausea, arthralgia, chemical pancreatitis, abdominal pain and cardiotoxicity. Amphotericin B is the drug of choice for Leishmaniasis but it has high nephrotoxicity (Chulay *et al*., 1985; Sundar *et al*., 2000).

1.5.3 Vaccines

Substantial efforts have been dedicated to the development of vaccines against *Leishmania* infection and crude or purified antigens have been shown to induce considerable levels of protection experimentally in susceptible animals. Vaccination is likely to be the main cost-effective measure to control Leishmaniasis (da Fonseca *et al*., 1997; Kedzierski, 2010). There are different types of vaccines namely DNA vaccines, recombinant protein vaccines, live attenuated vaccines and killed parasite vaccines which are described later.

1.6 Immune response to *Leishmania* parasites

Cummings *et al*., (2010) have shown that immunity against *Leishmania* infection is dependent on the development of strong T cell responses mostly of a Th1 type. Indeed, Th1 cells produce cytokines such as IFN-γ which activates macrophages

leading to the destruction of the parasite. Cytokines produced by Th2 cells such as IL-4 have the opposite effect and exacerbate the infection. Moreover, Verthelyi & Klinman, (2003) have shown that both CD8+ and CD4+ T-cells were essential for protection. The Th1 cytokine IFN-γ may up-regulate some antibodies such as IgG1 and IgG3 in humans, whereas the Th2 cytokines IL-4 and IL-5 stimulate the production of high levels of IgM, IgE, and IgG isotypes such as IgG4. A study on the *Leishmania* specific Ig isotypes and IgG subclasses in VL patient sera have detected elevated levels of IgM, IgE, IgG and IgG subclasses (Anam *et al*., 1999; Ryan *et al*., 2002). Ali *et al*., (2009) found that, Balb/c mice immunised with a *L. mexicana* gp63 DNA vaccine construct using a gene gun, increased IgG2a and IgG1 levels as early as 7 days after the immunisation. In addition, studies in mouse models have established that immunity is mostly dependent on the cell-mediated immune response and is influenced by the genetic background of the host (Mitchell *et al*., 1981; von Stebut & Udey, 2004). Intensive research in cutaneous Leishmaniasis caused by *L. major* has established a dichotomy in the T helper lymphocyte response (Kharazmi *et al*., 1999; Cummings *et al*., 2010). In resistant mouse strains such as C57BL/6, resistance to the *Leishmania* infections was associated with a Th1 response characterised by activation of T cells that produce IFN-γ. The expansion of the Th1 type CD4+ T cells is dependent on IL-12 (Afonso *et al*., 1994; Mattner *et al*., 1996). In these mice IFN-γ activates macrophages which assist in clearing the parasites. In contrast Balb/c mice, a susceptible mouse strain, respond to infection with a Th2-type response and high IL-4 levels (Alexander *et al*., 1999; Jones *et al*., 1998; Manna *et al*., 2008). This suggests that the genetic background of the host determines the disease outcome in these models (Fig 1.6).

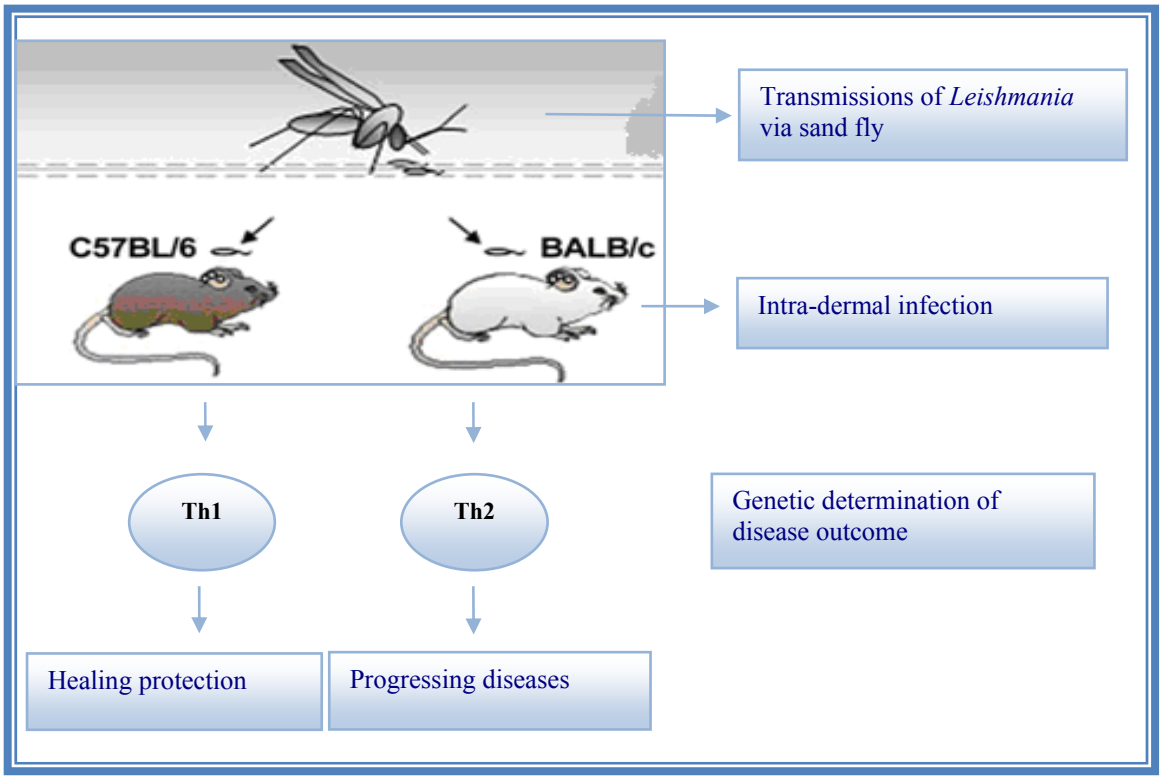


Figure 1.6 Th1/Th2 immune responses in *Leishmania* infection
Th1 immune responses observed in C57BL/6 mice is associated with Th1-dependent protective immunity. In contrast, Balb/c mice develop progressive lesions which is associated with a Th2-predominant immune responses (von Stebut *et al*., 2000).

While it is generally accepted that cellular, rather than humoral immunity, plays an important role in host defence against Leishmaniasis, a few studies have shown that antibodies are instrumental in providing resistance to many intracellular pathogens (Casadevall *et al*., 1998; Abass *et al*., 2007). The antibody response isotype profile depends on the cytokines created by antigen specific T cells. Furthermore, specific subclasses of immunoglobulin (IgG2a, IgG1) are associated with Th1 and Th2 responses respectively. It is known that IgG2a levels are regulated by IL-12 and IFN-γ, while IgG1 levels are controlled by IL-4, (Morris *et al*., 1994; Ramirez *et al*., 2010). However, this dichotomy is not absolute and it has been observed that IL-12 may enhance IgG1 production. It has been reported that IL-12 vaccinated mice developed strong Th1 responses and also showed significant increase in parasite specific antibody, particularly IgG1 isotypes. This suggests the possibility of IgG1

and IgG2a working in tandem rather than acting antagonistically (Wynn *et al*., 1996). Matthews *et al*., (2000) have shown that IL-13 is a key factor in shaping susceptibility to *L. major* infection. High expression of IL-13 in transgenic mice transforms the normally resistant C57BL/6 mouse to become susceptible to *L. major* infection even in the absence of IL-4 expression. This susceptibility is associated with a suppression of IFN-γ and IL-12 expression. Moreover, studies in Balb/c mice deficient in the expression of IL-4, IL-13, or both IL-13 and IL-4 showed that IL-13-deficient mice are resistant to infection and that there is an additive effect of deleting both IL-4 and IL-13. In addition, Chu *et al*., (2010) have shown that mice infected with *L. mexicana* have developed pathways to control host immunity, one of them is via the induction of IL-10 which has a suppressive effect on T-cell mediated IFN-γ through binding of antibody parasite complex to Fc Receptors for IgG (FcγRs), the cell surface receptors for IgG. To develop effective vaccine away from the destructive Ab responses, via the induction of IL-10, it is crucial to identify which isotypes of IgG that could exacerbate pathogenicity of the parasite to be avoided. It has been shown that IgG1 and IgG2a induce IL-10 from macrophages *in vitro* equally well but through different FcγR subtypes: IgG1 through FcγRIII and IgG2a mainly through FcγRI but also through FcγRIII. In contrast, mice deficient in IgG1 developed stronger and earlier IgG2a, IgG3, and IgM responses to *L. mexicana* infection and were more resistant to the infection. Thus, IgG1, but not IgG2a or IgG3, is linked to pathogenicity *in vivo*, and have been demonstrating that FcγRIII is required for the progression towards the chronic phase of the disease.

a) Th1/Th2 in *Leishmania* infection

Immune responses in Leishmaniasis were intensively investigated in humans, susceptible and resistant animal models. Ehrchen *et al*., (2010) have shown, using *L. major* infection, an activation of the Th1 and Th2 in resistant and susceptible mice respectively. Gene expression analysis performed on samples obtained from infected skin of resistant and susceptible mice following parasite inoculation clearly demonstrated an upregulation of genes linked to either Th1 or Th2 responses respectively. Similar Leishmaniasis Th1/Th2 dichotomies were also observed in humans. In a study on 14 volunteers with a history of self-healing cutaneous Leishmaniasis compared with 18 healthy control volunteers, the profile of IL-5, IL-10, IL-13 and IFN-γ cytokines produced by purified CD4+/CD8+ T cells in reaction to *Leishmania* antigens was analysed. The results have shown that all the volunteers have maintained long-term immune responses against *Leishmania* antigens as determined by *Leishmania* skin test and IFN-γ production providing evidence of the existence of Th1 responses (Rostami *et al*., 2010). Jiaxiang *et al*., (2005) have investigated the role of regulatory T cells in controlling Th cell activities. High ratios of $CD4^{+}CD25^{+}CD86^{+}$ T cells were detected in the draining lymph nodes as well as skin after one to three weeks of parasite inoculation indicating local accumulation of T regulatory cells with high levels of FoxP3, TGF-β and IL-10RI transcripts. The beneficial effect of T regulatory cells for parasite survival was temporary and connected with inhibition of IFN-γ producing effector T cells. This study has demonstrated the intriguing role of T regulatory cells in restraining pathogenic potential of *Leishmania* parasite during nonhealing *Leishmania* infection and highlighted a balance between T regulatory and T effector cells in determining the outcome of cutaneous *Leishmaniasis*.

1.6.1 Innate immune response to *Leishmania* parasites

1.6.1.1 Natural killer cells

Leishmania induces CD1d-dependent activation of NK T cells in wild type animals and increases susceptibility of NK T cell deficient $CD1d^{-/-}$ mice to *Leishmania donovani* infection. The elicited response, occurring as early as 2 hours after infection, was Th1 polarized and IL-12 independent. The *Leishmania* surface glycoconjugate Lipophosphoglycan (LPG) and related glycoinositol phospholipids bound with high affinity to CD1d and induced a CD1d dependent IFN-γ response in naïve intrahepatic lymphocytes. Both these data recognize *Leishmania* surface glycoconjugates as possible glycolipid antigens responsible for protection in this model, suggesting an important role for the CD1d–NK T cell immune axis in the early response to infection with visceral *Leishmania* (Amprey *et al*., 2004). It has also been shown that naive natural killer (NK) cells are important in *Leishmania* infection as a source of IFN-γ together with macrophage with the potential to trigger the Th1 immune response in cutaneous Leishmaniasis (Scharton & Scott, 1993; Scott 1991). In addition, using the mutant beige mice with low NK activity, the direct importance of NK cells in the development of visceral Leishmaniasis has been shown in mice with an intermittent suppression or depletion of NK cells by anti-asialo GM1 or anti-NK1.1 monoclonal antibodies, which resulted in an increased susceptibility of mice to *Leishmania major* (Kirkpatrick *et al*., 1985; Laskay *et al*., 1993). Sanabria *et al*., (2008) have shown the importance of the interaction between NK and DCs in the development of anti-viral and anti-tumour immune responses. They developed a DC-NK cell co-culture system to study the role of NK cells in modulating the functions of *Leishmania* infected DCs. They found that addition of freshly isolated NK cells

and/or resting NK cells significantly increased the activation of DCs that were pre-infected with *L. amazonensis* promastigotes. These activated DCs in turn stimulated NK cell activation mainly by cell contact-dependent mechanisms.

1.6.1.2 Macrophages in *Leishmania* infection

Macrophages play a critical role in resistance against a number of pathogens. These cells are not only involved in the initiation or maintenance of cell-mediated immune responses (Zhu *et al*., 2009), they also act as effector cells, functions that are linked to their ability to present endogenous and exogenous antigens (Ag) in the context of MHC class I and class II molecules, and to generate potent microbicidal molecules (Auger *et al*., 1991). The macrophage is the major host for the *Leishmania* parasite (Colotta *et al*., 1992). Activation of macrophages contributes to the successful elimination of the parasite. This occurs by production of toxic oxygen metabolites including nitric oxide (NO), super oxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Assreuy *et al*., 1994). A diversity of stimuli is able to make the morphological, biochemical and functional changes characteristic of activated macrophages. Activated macrophages produce different cytokines like TNF-α, IL-6, IL-18, IL-12 and IFN-γ (Hirohashi & Morrison, 1996; Munder *et al*., 1998; Gee *et al*., 2009). IL-12 and IFN-γ are effective adjuvants and there is a Th1 type of immune response in most intracellular parasitic infections (Afonso *et al*., 1994; Ezra *et al*., 2010). The main producers of IL-12 are antigen presenting cells, such as macrophages and dendritic cells (Yamane *et al*., 1999), which produce IL-12 through CD40/CD40L interactions (Kato *et al*., 1996). The nitric oxide production is the final common pathway involved in the destruction of *Leishmania* parasites by macrophages. Inhibition of NO production has been shown to render macrophages more susceptible

to *L. major* infection, thus being unable to interfere with the survival of the parasite *in vitro* (Assreuy *et al*.,1994; Green *et al*., 1990). The role of IFN-γ in inducing NO production in macrophages was demonstrated and found to independently enhance iNOS transcription and NO release from stimulated mouse peritoneal macrophages (Ding et al., 1988; Khoshdel *et al*., 2009). However, a number of cytokines have been shown to enhance NO production synergistically with IFN-γ thus potentially mediating parasite control *in vivo*. IFN-α is the most extensively studied anti-*Leishmania* cytokine, which synergizes with IFN-γ in the induction of iNOS and NO production by macrophages *in vitro* (Green *et al*., 1990; Moll *et al*.,1990, Rogers *et al*., 2009).

1.6.1.3 Neutrophils in Leishmaniasis

Polymorphonuclear leukocytes (PMNs) are considered to be the main effector cells in infection inducing inflammatory reactions in which they serve to destroy invading pathogens (Witko-Sarsat *et al*., 2000). There is increasing evidence to suggest that *Leishmania* pathogens even infect and develop within neutrophils. Taking *L. major* as an intracellular pathogen, PMNs are manipulated in such a way that the pathogens are able to use the granulocytes as host cells. The capability to maintain and survive infectivity in PMNs subsequently enables these organisms to establish productive infection (Laskay *et al*., 2003). Following experimental skin infection with *L. major,* a local inflammatory process was immediately initiated and a few hours later a wave of PMNs was shown to migrate into the skin. Two or three days later on, the second wave of cells to enter the site of infection were monocytes and macrophages, (Muller *et al*., 2001; Sunderkotter *et al*., 1993). In addition to its chemokine activity, IL-8 also activates other cell functions of PMNs such as phagocytosis (Scapini *et al*., 2000).

The *Leishmania* organism induces the production of IL-8 by PMNs, thus increasing the infiltration speed of the neutrophils to the site of infection and assists uptake of the parasites. IL-8 is the main chemokine not only in the skin but also at other sites of entrance used by pathogenic microorganisms (Molestina *et al*., 1999). Carmo *et al*., (2010) have shown that the co-culturing of infected macrophages with normal neutrophils enhanced the destruction of the parasites which did not require a direct contact between infected macrophages and neutrophlis. It was also shown that parasite clearance by macrophages did not involve the classical activation pathway by TNF-α, as reported for other *Leishmania* species.

1.6.1.4 Eosinophils in to Leishmaniasis

Cutaneous Leishmaniasis which induces skin lesions is characterized by a granulomatous inflammation in the subcutaneous tissue and the dermis. Eosinophils are found in the granuloma as the main constituent of the inflammatory cells, which are also found to be associated with the induction of IL-5 (Boom *et al*., 1990). Eosinophil chemotactic activity has been reported in lysates of *L. amazonensis* promastigotes, *in vitro* and *in vivo*. The lysate use specific chemotactic activity on eosinophils without the contribution of complement activation. Saito *et al*., (1996) and Driss *et al*., (2009) have shown that tissue eosinophilia was observed shortly after intraperitoneal inoculation of *L. amazonensis* in the subcutaneous tissue of mice Elshafie *et al*., (2010). Also intraperitoneal inoculation of lysed promastigotes from five types of *Leishmania* species (*L. donovani*, *L. tropica*, *L. chagasi L. braziliensis* and *L. amazonensis*) induced eosinophil accumulation in the mouse peritoneum. This eosinophil infiltration was detected in $C5^{-/-}$ $lack^{-/-}$ AKR mice C57BL/6, indicating complement independent eosinophil chemotaxis by the parasite. In an *in vitro* study,

Watanabe *et al*., (2004) have shown that the progress of *L. amazonensis* infection in IL-5 transgenic mice in which 50% of peripheral blood leukocytes are eosinophils was significantly suppressed. Immunisation by electroporation into muscle of Balb/c mice with IFN-γ, IL-4 & IL-5 constructs was also investigated; the IL-5 plasmid was most effective in slowing down the progress of the infection in comparison with the other two plasmids. Immunisation with IL-4 plasmid had little effect where IFN-γ had no effect on the progress of disease. Thus, IL-5 gene transfer into muscle by electroporation was helpful in protection against *L. amazonensis*.

1.6.1.5 Dendritic cells at the interface of innate and acquired immunity

DCs are essential in innate immunity and in the initiation of adaptive immunity (Banchereau & Steinman, 1998; Behar *et al*., 2010). The restriction of an adaptive immunity pathway by innate immunity is dependent on unique DC functions and on the type of DCs resulting from the effect of chemokines and cytokines (Wen *et al*., 2008). DCs contribute in cell mediated immunity by taking up antigen and presenting peptide on MHC molecules (Trombetta & Mellman, 2005). DC activation by pathogen-derived molecules is initiated by a switch in chemokine receptor expression that leads to migration to draining lymph nodes in a chemokine receptor-dependent manner. The up-regulation of costimulatory CD80, CD86, and CD40 and MHC molecules, alongside secretion of chemokines and cytokines, assist DC antigen presentation to naive T cells with an antigen-specific receptor (Banchereau *et al*., 2000; Leon *et al*., 2007). Infection with *Leishmania* induced inflammatory responses involving a strong increase in numbers of DCs at the infection site and draining lymph nodes (dLNs). Also during *Leishmania* infection, monocytes are recruited to the dermis and differentiate into "dermal monocyte derived DCs", which then migrate

into the dLNs. In addition, monocyte recruited to the dLNs subsequently differentiate into LN "monocyte-derived DCs", the kinetic studies of monocyte differentiation into DCs during *Leishmania* infection that lead to specific T cell stimulation have suggested that dermal monocyte derived DCs eventually control the initiation of defensive Th1 responses against *Leishmania*. Thus, the demonstration of monocyte differentiation potential into DCs during *in vivo Leishmania* infection and of local DC differentiation in inflammatory foci propose that *de novo* formed monocyte derived DCs are important in T cell immunity against pathogens (Leon *et al*., 2007).

1.6.1.6 Toll-Like receptors (TLR)

One of the first protective systems against invasive microorganisms is the TLR signalling pathway (Oda & Kitano, 2006). TLRs are transmembrane proteins that are specifically expressed on cells mediating "Natural" immunity and are responsible for recognition of invasive pathogens causing human disease. The TLR family presently consists of 11 members, which have specificity for different pathogens and trigger the induction of different cytokines (Janssens & Beyaert, 2003). TLRs are located on either the internal membranes or plasma membrane of macrophages, NK and DCs cells. B and T lymphocytes also express TLRs. The TLR's cytoplasmic signalling domains are separated from the ligand recognizing extracellular or internal membranes by a single membrane spanning domain (Sarkar *et al*., 2007; Sarkar *et al*., 2005). NF-κB becomes activated, after the binding of TLRs to their specific pathogen antigens, and is transported to the nucleus where it triggers the transcription and synthesis of pro-inflammatory cytokines (Barton & Medzhitov, 2003). These specific pathogen antigens are called pathogen-associated molecular patterns (PAMP) and in general are internal structures or surface molecules (proteins, RNA, DNA, and

enzymes) produced by microbes but not host cells. These PAMP antigens enable the innate immune system to respond immediately to invading microbes (DiDonato et al., 1996; Ghosh & Bandyopadhyay, 2004). There is an important step between the activation of TLR and the release of NF-κB, which is mediated by adaptor molecules. MyD88 is the most common adaptor molecule for the activation of NF-κB and is present in most TLRs (Fig 1.6.1.6) (Ohnishia *et al*., 2009). Signal transduction leading to TLRs activation is complex where the transcription factor NF-κB plays a vital role in the induction of inflammatory mediators. The activation of the transcription factor NF-κB requires both phosphorylation and degradation of the IκB. As part of TLRs signalling cascades, phosphorylation dependent degradation of IκB is an important step in the activation of NF-κB (Janssens *et al*., 2003).

a) TLR pathway and Leishmaniasis

Several studies have confirmed that different receptors mediate the uptake and phagocytosis of *Leishmania* by macrophages. One of the first studies evaluating TLRs and the MyD88-dependent pathway in *Leishmania* infection was performed by (Hawn *et al*., 2002). The *Leishmania major* infection and cytokine expression in macrophages in $MyD88^{-/-}$ mice was investigated. There was a decreased amount of mRNA expression of IL-1α in the $MyD88^{-/-}$ mice as well as an activation of the IL-1α promoter in the $MyD88^{+/+}$mice. This was similar to the levels found with lipopolysaccharide (control). By studying the TLRs in a C57BL/6 mouse model (Debus *et al*., 2003; Muraille *et al*., 2003) the importance of the MyD88 in the (resistance model) Th1 response to cure *Leishmania* cutaneous lesions has been shown. $MyD88^{-/-}$ mice had an increased number of cutaneous lesions compared with wild-type C57BL/6 mice ($MyD88^{+/+}$).

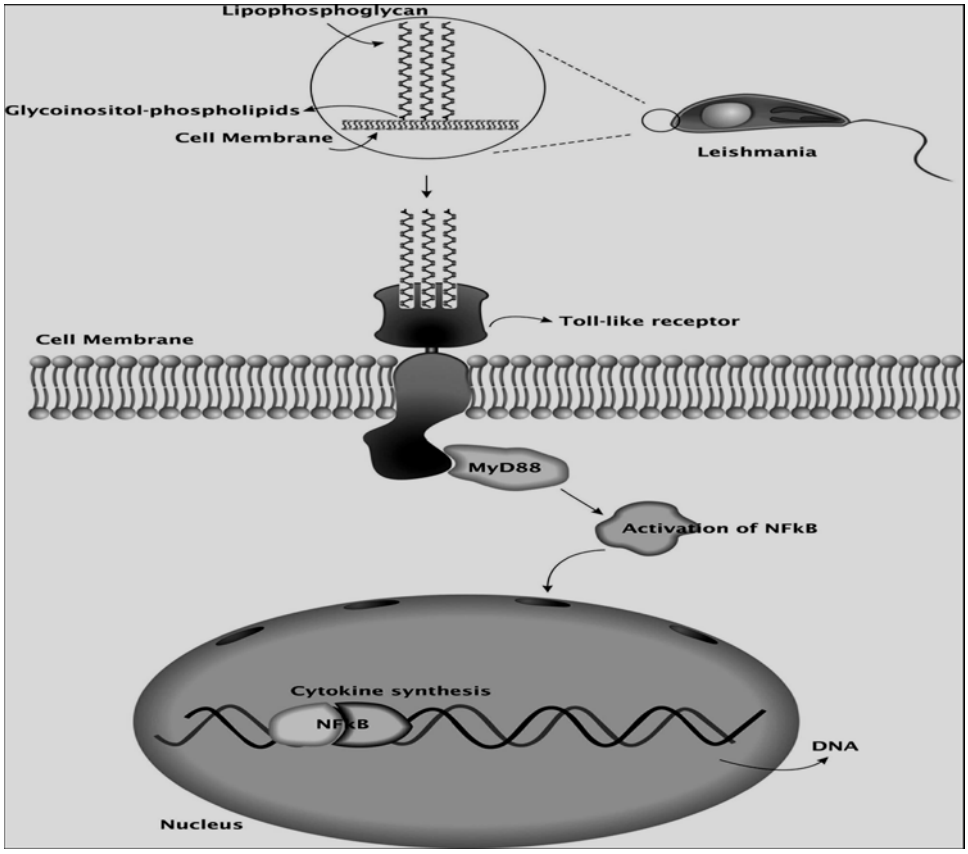


Figure 1.6.1.6 TLR and MyD88 pathway in *Leishmania* infection
Binding of TLR with pathogen-associated molecular patterns (PAMP) which is the LPG from *Leishmania* parasite initiates signalling cascades that involves a number of proteins, such as MyD88. These signalling cascades lead to the activation of transcription factors, such as NF-κB and inducing the secretion of pro-inflammatory cytokines and effector cytokines that direct the adaptive immune response pro-inflammatory cytokines (Tuon *et al*., 2008).

The number of lesions of the MyD88$^{-/-}$ mice was similar to that in Balb/c mice, which have a dominant Th2 response and a tendency to have an increased number of *Leishmania* lesions. An increase in IL-4 levels and decrease in IFN-α and IL-12(p40) levels were also recognized. In addition de Veer *et al*., (2003) demonstrated the relationship of LPG to MyD88 and TLR2. The LPG was linked with an increased level of TNF-α independent TLR4, where NF-kB activation by LPG was mediated by TLR2. TLR4 is essential for parasite control, possibly due to the activity of iNOS, leading to NO synthesis and *Leishmania* death (Antoniazi *et al*., 2004; Kropf *et al*., 2004). It has been shown that LPG has no effect on TLR4 (de Veer *et al*., 2003; Debus *et al*., 2003). Furthermore, IL-12 is a very important cytokine in the immune

response against *Leishmania* and can be activated by TLR9. The production of pro-inflammatory cytokines, specially IL-12 by TLR9 was observed in infected mice with *L. major* (Li *et al*., 2004). TLR9 is expressed only on DC and B lymphocytes in humans. NK cells in visceral Leishmaniasis are associated with good protection, and TLR9 is necessary for the activation of these cells as it is essential for the production of IL-12 by DCs (Schleicher *et al*., 2007). TLR3 also contributes to the detection of *Leishmania* (Flandin *et al*., 2006). In addition, receptors such as TLR7, TLR8, and TLR9, situated in intracellular endosomal membranes, recognize double-stranded RNA, leading to the production of IFN-γ and trigger NF-kB (Alexopoulou *et al*., 2001). The localization of TLR3 induces cytokine production by means of a signaling pathway (Hoebe *et al*., 2003). On the other hand, TLR3 also uses a MyD88-independent pathway to NF-kB and production of IFN-γ (Flandin *et al*., 2006; Ohnishia *et al*., 2009). The production of TNF-α and the activation of NF-kB that occurred in experiments with *L. major* were similar to results obtained with other *Leishmania* species, such as *L. mexicana*, *L. aethiopica*, and *L. tropica* (de Veer *et al*., 2003). Muraille *et al*., (2003) found that the MyD88 dependent TLR pathway is involved in the induction of DC maturation, which was established previously with *L. major* (Table 1.6.1.6).

Model	Species	Pathway or TLR	Outcome
Balb/c	*L. major*	MyD88$^{-/-}$	Decreased IL-1α promoter activation
C57BL/6 (B6WT)	*L. major*	MyD88$^{-/-}$	Increased number of lesions and IL-4 levels, decreased levels of IFN-α and IL-12
Balb/c	*L. major*	MyD88$^{-/-}$ and IL-4^{-}	Inhibition of IL-4, increased IFN-γ levels
C57BL/6	*L. major*	MyD88$^{-/-}$	Absence of TLR2 increased the number of lesions
Cell culture	*L. major*	TLR2	LPG could activate NF-kB by TLR2 ligation
C57BL/10ScN	*L. major*	TLR4$^{-/-}$	Absence of TLR-4 increased the number of lesions
C57BL/10ScN	*L. major*	TLR4$^{-/-}$ but IL-12	Leishmaniasis control was TLR4 and IL-12 dependent
Balb/c	*L. major*	TLR9	Vaccine decreased the number of lesions by the TLR9 pathway
C57BL/10ScN	*L. major*	TLR4^{-}and TLR4^{+}	Little variation of chemokine levels
Cell culture	*L. donovani*	TLR3	TLR3 was activated by double-stranded RNA
Balb/c	*L. major*	TLR9	IL-18 improved the Th1 response, probably via TLR9
C57BL/6	*Leishmania*	MyD88	Pathway improved DC maturation
C57BL/6 Balb/c	*L. major*	TLR4	Neutrophil elastase activated TLR4
C57BL/6, Balb/c	*L. infantum*	TLR9$^{-/-}$	Cytokine production from DCs was dependent on TLR

Table 1.6.1.6 TLR pathway and Leishmaniasis
MyD88 is the most common adaptor molecule for the activation of NF-κB and is essential for activation of most TLRs. MyD88-/- mice had an increased number of cutaneous lesions compared with wild-type C57BL/6 mice (MyD88+/+) and TLR responsible for recognition of invasive pathogens causing human disease (Tuon *et al*., 2008).

1.6.2 Adaptive immune system

1.6.2.1 Dendritic cells as antigen presenting cells

DCs form a family of leukocytes that play critical roles in the innate and adaptive immune systems. It has also been shown that DCs are the source of different

cytokines such as IL-12, IL-10 and IFN-γ that are involved in the induction of a different type of T cell (Cella *et al*.,1996; Qi *et al*., 2003; Stober *et al*., 2001). It has been reported that incubation of *Leishmania* promastigotes with dendritic cells induced early IL-12 production *in vitro*. In addition, DCs play an active role in the initiation of the T cell immune response during *Leishmania* infection (Guermonprez *et al*., 2002; Donaghy *et al*., 2010*)*. Activation pathways induced by DCs are dependent on their differentiation stage and lineage. For example, immature, non-activated antigen-loaded DCs either induce anergy of specific T cells or the development of regulatory T cells that prevents the activation of T effector cells. At this stage, the plasma membranes of DCs display fewer MHC class I and MHC class II molecules and either none or very few co-stimulatory molecules. In contrast, mature activated antigen-containing DCs display high levels of MHC class I, MHC class II and co-stimulatory molecules on their cell surfaces, which leads to activation of T cells (Berberich *et al*., 2003). DCs rather than macrophages are more likely to prime T cell responses against *Leishmania* infection to induce protective immunity, since infection of macrophages with this parasite does not normally lead to their activation, migration or induction of IL-12. DCs phagocytose *Leishmania* amastigotes or free antigens after they are released into the tissue (von Stebut & Udey, 2004). This may lead to DC activation, up regulation of MHC class I and II, as well as co-stimulatory molecules and migration to the draining lymph nodes where they present *Leishmania* antigen to Th0 cells while releasing IL-12, thus inducing Th1 development (Fig 1.6.2.1).

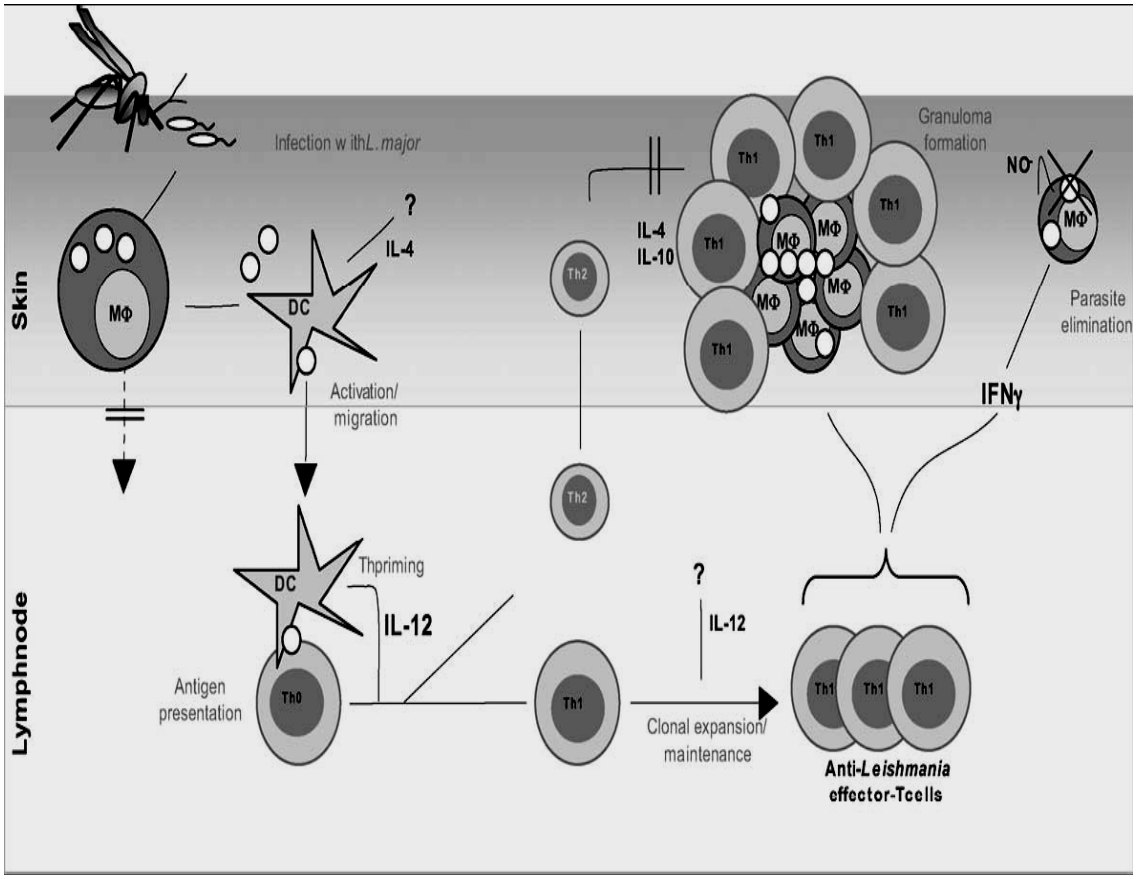


Figure 1.6.2.1 DC presents *Leishmania* antigens
DCs prime T cell responses against *L. major* and induce protective immunity. DCs phagocytose *Leishmania* amastigotes or free antigens after they are released into the tissues. This may lead to DC activation and migration to the draining lymph nodes where they present *Leishmania* antigen to Th0 cells while releasing IL-12, thus inducing Th1 development (von Stebut & Udey, 2004).

1.6.2.2 MHC class I and class II expression in *Leishmania* infection

The main factor limiting T cell responses to self-antigens is the regulated expression of co-stimulatory molecules (Kaye, 1995; Guerin *et al*., 2009). T cell activation depends on two signals: TCR ligation with MHC antigens provides signal (primary activation signal or signal 1) mediated via the activation of protein tyrosine kinases and requiring the CD45 protein tyrosine phosphatase. Signal 2 is achieved by the interaction of a diversity of receptor- counter-receptor pairs, of which the best characterized are those involving CD80/CD86 on APC and CD28/CTLA-4 on T cells, which involve biochemical pathways distinct from those initiated by TCR complex

activity (June *et al*., 1994; Benedict *et al*., 2007). Moreover, Lang *et al*., (1994) found that *in vivo,* macrophages infected with *Leishmania* are able to present antigens to *Leishmania s*pecific CD4+ and CD8+ T lymphocytes, which recognize the processed antigens bound to MHC class II and MHC class I molecules respectively. Infected macrophages and their role in parasite lysis suggest that these cells are the central APC-mediating parasite control mechanism (Alexander & Russell, 1992; Bosschaerts *et al*., 2010). Production of TNF-α, IL-12, (NO) and IFN-γ by macrophages can contribute to Th1 polarization, effector Th1 CD4+ T cell maintenance, and parasite lysis (Diefenbach *et al*., 1998; Richard *et al.*, 2010). Macrophage secretion of these molecules can be facilitated by MHC II peptide-TCR and CD40-CD40L interactions (Campbell *et al*.,1996; Kamanaka *et al*.,1996; Rao, 2001). Kwan *et al*., (1992) have shown that during the activation of macrophages, among the important responses to IFN-γ are the increased expression of MHC complex class II and class I genes. According to Lemos *et al*., (2004) the control of the intracellular protozoan, *L. major*, requires MHC class II dependent antigen presentation and CD4+ T helper cell 1 differentiation. However, the *Leishmania* lesions also contained infected DCs, which may directly help in the development of protection in two ways. First, infection of DC with *L. major* would lead to activation of the T cells with upregulation of MHC class I and II expression (a scenario that proved incorrect in many cases) as well as costimulatory molecules and migration to the draining lymph node, leading to priming of T cells. Second, cytokines released by DC are essential for Th1 differentiation (Belkaid *et al*., 2000; Mattner *et al*., 1996; von Stebut *et al*., 2000). CD8+ T lymphocytes are an important component of the protective immune response developed in *Leishmania* infected mice. *Leishmania* are always located in membrane-bound compartments belonging to the endocytic pathway, which raises the question

of the proximity of *Leishmania* Ags and MHC class I molecules. Indeed, until recently it was generally thought that complexes between processed antigens and MHC class I molecules were formed exclusively in one or several compartments of the biosynthetic pathway of the APC. Several recent findings however, are also consistent with a peptide loading of MHC class I molecules in endocytic compartments. The constitutive endocytosis of cell-surface MHC class I molecules has been described in several cell types (Vega & Strominger, 1989; Basha *et al.*, 2008) and macrophages, and perhaps also dendritic cells, seem to be endowed with a unique capability to present exogenous Ags in association with MHC class I molecules (Debrick *et al*., 1991; Pfeifer *et al*., 1993).

1.6.2.3 CD4+ T cells in the immune response to *Leishmania* parasite

T cell mediated immunity plays a central role in the host response to control intracellular pathogens. The *Leishmania* infection induces an increase of the T cell response to different species of the parasite (*L. donovani L. amazonensis* and *L. braziliensis*), and an increase in levels of IL-2 and IFN-γ production (Gabaglia *et al*., 2000; Cummings *et al*., 2010). Studies on T cell activities in Leishmaniasis are likely to have a great impact on the understanding of the disease in humans, and assist successful vaccine development. This depends on the applicability of these results to human disease and on continued studies in murine models to further understanding of the mechanisms involved in the generation and maintenance of central memory and effector memory (Gollob *et al*., 2005; Kaech *et al*., 2002). In a study by Ramer *et al*., (2006) a comparison of draining lymph node cells from *L. major* and *L. amazonensis* infected mice at 10 weeks post infection showed equal percentages of memory and effector phenotype CD4+T cells that produce IL-2 and proliferate after

antigen stimulation. In other studies IL-13 and IL-4 were detected in the skin after initial lesion development, suggesting that Th2 cytokines play an immuno-regulatory role in early infections (Bourreau *et al*., 2003; Murray *et al*., 2006). However, cure of the infection was regularly associated with the production of IFN-γ only, while IL-10 was present in persisting lesions (Rogers *et al*., 2002). In addition, treatment of non-healing cutaneous Leishmaniasis with IFN-γ resulted in the rapid and complete resolution of lesions (Kolde *et al*., 1996; Nylen *et al*., 2010).

1.6.2.3.1 Chemokines in *Leishmania* infections

Chemokines are a growing group of chemoattractant cytokines that play important roles in physiological and pathological processes. Successful immunity to *Leishmania* depends on recruitment of suitable immune effector cells to the site of infection and chemokines play an essential role in the process. *Leishmania* parasites possess the ability to modify the chemokine profiles of their host, thereby facilitating establishment of progressive infection. They are single polypeptides of about 67 to 127 amino acid residues in length (Moser & Willimann, 2004). Some chemokines have been shown to regulate cell differentiation (Gu *et al*., 2000), and distinct patterns of chemokine secretion have been observed in differentiated cells (Muller *et al*., 2003). About 50 human chemokines and 20 chemokine receptors have been recognized up to now (Viola & Luster, 2008). *L. major* has been shown to actively modify the chemokine profile of the infection site and thus recruit cells that will favour the development of persistent infection (Katzman & Fowell, 2008). An important example is LPG, the most abundant glycolipid on the surface of *Leishmania* promastigotes, which inhibits the production of CCL2 also known as monocyte chemotactic protein-1 (MCP-1) by endothelial cells thus affecting

monocyte transendothelial migration (Lo *et al*., 1998; Oghumu *et al*., 2010). Moreover, *Leishmania infantum* infection of human macrophages causes a down regulation of the chemokine receptor CCR1 which could potentially restrict macrophage recruitment to infected tissues, thereby allowing parasite progression (Panaro *et al*., 2004). In contrast, as several studies have shown, chemokine and chemokine receptor expression by *Leishmania* infected host cells could be a means of facilitating the hosts' ability to restrain the parasite to the site of inoculation and mount an effective immune response (Matte & Olivier, 2002). Muzio *et al*., (2000) have shown that *L. major* infected mice induce overall upregulation of CCL5, CCL3, CXCL10 and CCL2 in the footpads and LNs, whereas these chemokines are constitutive in the spleens of TLR4-competent and deficient mice. However, the expression patterns are not affected directly by the presence or absence of TLR4 (Antoniazi *et al*., 2004). The parasite itself also produces a chemoattractant protein called *Leishmania* chemotactic factor, which can attract PMNs (van Zandbergen *et al*., 2002). PMNs are the first cells to arrive at the site of *Leishmania* infection. In humans, PMNs containing *Leishmania* start secreting chemokines such as IL-8 that are essential in attracting more PMNs to the site of infection (Laufs *et al*., 2002). The level of chemokine mRNA was measured in *L. major* infected ears during the first 48 hours post infection. In C57BL/6 infected mice, CCL3 mRNA was strongly induced within 24 hours of *L. major* inoculation, while significantly less CCL3 mRNA was induced in *L. major* infected Balb/c mice. *L. major* induced only a small increase in CCL4 and CCL5 mRNA at the site of infection, while infection did not induce CCL20 mRNA (Charmoy *et al*., 2010).

1.6.2.4 CD8+ T cells immune response to *Leishmania* infection

CD8 T cells are essential in the defence against viruses, yet little is known of their participation in the host defence against parasites, such as *Leishmania*. Mouse models of Leishmaniasis studies suggest that CD8 T cells play a significant role in protection through IFN-γ production and patients infected with various *Leishmania* strains where CD8 T cell cytotoxicity and apoptosis of autologous *Leishmania* infected macrophages correlate with cure. Dendritic cells activate CD8 T cells through antigen presentation (Ruiz & Becker, 2007) and T-cells producing IFN-γ may activate macrophage (Chan, 1993; Tierney *et al*., 2009). Obligatory intracellular parasites such as *Leishmania* species, can deliver antigens to the host cell cytoplasm that are presented through MHC class I molecules to protective CD8 T cells. The *in vivo* conditions of specific CD8 T cell activation during natural infection are not fully known, but the anti-parasitic mechanisms mediated by CD8 T cells may include both IFN-γ dependent and independent pathways. CD8 T cells are strong inhibitors of growth, which has prompted several investigators to consider whether stimulation of T cells can be a feasible strategy for the development of effective subunit vaccines against these parasitic diseases (Bankoti & Stager, 2010). Estimation of the lymphocyte proliferation response produced *in vitro* by *L. braziliensis* antigens has been shown. *L. braziliensis* specific T cells stimulated *in vitro* for 5 days were collected and typed for CD4+ and CD8+ cells and the results before and after therapy were evaluated. Leishmaniasis patients demonstrated an enhanced CD8+ T cell response and a decrease in the proportion of CD4+ T cell blasts in culture. The levels of IFN-γ in T-cell culture supernatants showed a tendency to increase in cured patients. These results confirm a pattern of high proportions of *Leishmania* reactive CD8+ T cells and low proportions of *Leishmania* reactive CD4+ T cells after cure

(Fig 1.6.2.4) (Da-Cruz *et al*., 1994). As similar result was reported in patients with a history of self-healing cutaneous Leishmaniasis (Rostami *et al*., 2010), the activation of $CD8^+$ cells was also reported by *in vitro* treatment with an influenza virus-like particle (VLP) vaccine in a DC T cell co-culture system. VLP pulsed DCs were co-cultured with autologous $CD8^+$ T cells from 5 donors. $CD8^+$ T cells activation was assessed using cell surface and intracellular cytokine staining. The percentage of activated $CD8^+$ cells was investigated in 4 of the 5 donors. VLP influenza vaccine was shown to stimulate $CD8^+$ T cells using DC antigen presentation, possibly through the MHC class I pathway (Song *et al*., 2010).

CD8 cytotoxic T lymphocyte kills target cell specifically via different mechanisms such as releasing a mixture of granzymes and perforin. Perforin is a pore-forming protein that initiates the killing of target cells by inserting itself in their membranes causing necrosis. The first sign of perforin induced necrosis is the swelling of organelles which then followed by disruption of cytoskeleton leading to complete lysis of the target cell. Granzymes on the other hand induced killing of target cells by apoptotsis of target cells. The other mechanism is through Fas/APO ligand (CD95 ligand) which is expressed on CTLs and interaction with corresponding receptor on target cells triggers downstream death message leading to apoptotsis. Fas/APO ligand induced apoptotsis is indistinguishable from that induced by granzymes (Groscurth and Filgueira, 1998).

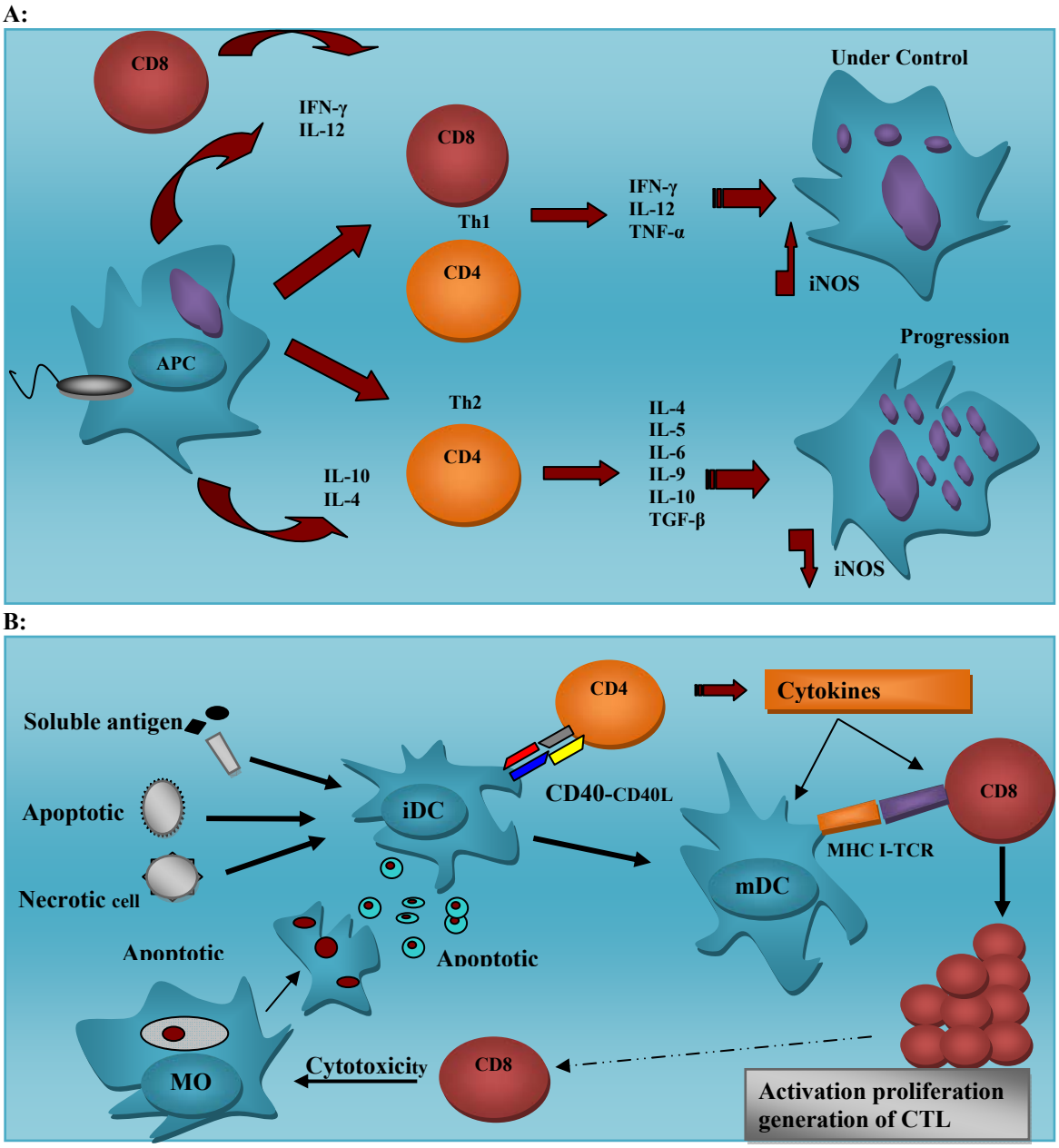


Figure 1.6.2.4 Immune responses to *Leishmania*
A: Susceptibility or resistance to Leishmaniasis depends on the types of secreted cytokines. Whereas a Th1 response leads to parasite destruction, a Th2 response leads to parasite survival and disease progression**. B:** Activation of T cells by cross-presentation, immature dendritic cells (iDC) take up antigens, such as soluble antigens, necrotic or apoptotic cells, which are processed and presented to CD4 T cells in association with costimulatory molecules. The interaction CD40–CD40L activates CD4 T cells, which produce cytokines that activate dendritic cells (DC) as well as CD8 T cells. The mDC present diverse *Leishmania* peptides through MHC class I to CD8 T cells, inducing their activation and proliferation into effector cells (Ruiz & Becker, 2007).

1.6.3 Humoral immunity

1.6.3.1 Complement activation in Leishmaniasis

The complement system has three main roles in host immunity: opsonin, lysis of target cells or microbes and activation of phagocytes (Fig 1.6.3.1). The **classical** complement **pathway** is activated via immune complexes. The **alternative** complement **pathways** are phylogenetically older and proceed through the lack of antibody. The **lectin mediated** pathway constitutes the binding of a serum lectin, such as mannan-binding protein (MBP), to target molecule, in addition to the early components of the classical pathway which include C1, C4, and C2. Each pathway leads to the arrangement of a C3 convertase, a C5 convertase, and a membrane attack complex (MAC) (Brittingham & Mosser, 1996).

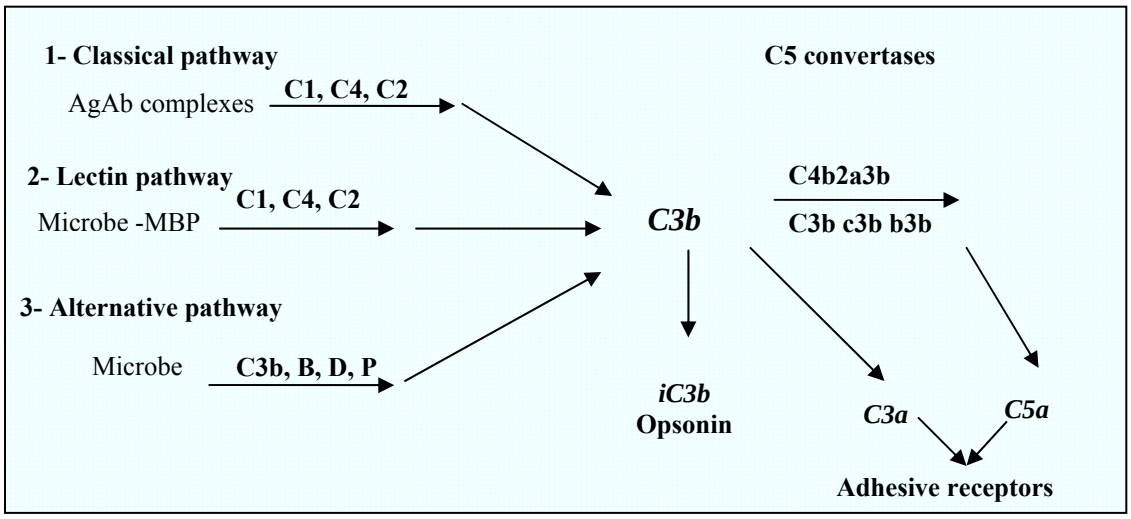


Figure 1.6.3.1The Complement System
There are three types of complement activation pathways; the classical pathway which is dependent on antigen antibody complex, the lectin pathway which is initiated through binding microbe with MBP, and the alternative pathway where binding to microbe initiates this process. All pathways leads to the arrangement of a C3 convertase, a C5 convertase (Brittingham & Mosser, 1996).

The complement system is comprised of a complex network of activators, regulators, effector mechanisms and signals. The mechanism of complement activation by promastigotes revealed that *Leishmania* can activate complement by the alternative pathway (Brittingham & Mosser, 1996, Lieke *et al*., 2008). Complement activation by *L. donovani* and the metacyclic stage *L. major* promastigotes may also involve components of the classical pathway. According to previous studies naturally occurring antibodies play a major role in the activation of the classical complement pathway by *L. donovani* (Bandyopadhyay *et al*., 2004), other studies suggest that the lectin-mediated pathway of complement activation is another major pathway of the complement system activated by *Leishmania* (Green *et al*., 1994; Olivier *et al*.,2005; Alonso *et al*., 2007). The two most abundant *Leishmania* surface molecules, LPG and gp63 participate in the activation of complement, as do all strains and species of *Leishmania* studied to date. Researchers observed that the majority of C3 fixed to the surface of *L. major* promastigotes was bound to LPG (Planck 2000; Soares *et al*., 2010). They indicated that purified LPG can compete with intact promastigotes for complement fixation (Mosser *et al*., 1992). The transfection of mammalian cells with gp63 could easily be converted to efficient activators of complement by the expression of this protein on the surface (Brittingham *et al*., 1995). The complement system is the most important defence mechanism of the innate immune system, which helps to clear pathogens from the organism by disrupting the plasma membrane of the target cells. Metacyclic promastigotes of *Leishmania* can activate complement using both the classical and the alternative pathways. Opsonization of *Leishmania* metacyclic promastigotes with complement is fast and lysis by the membrane attack complex (C5b–C9 complex) following serum contact is 90% efficient in killing all immunised parasites in 3 min. Interestingly, *Leishmania* have evolved to resist and

circumvent full complement lysis with many mechanisms (Dominguez *et al*., 2003). Procyclic and metacyclic *L. major* promastigotes are more resistant to complement lysis compared to the amastigotes. This is mediated through alteration of a membrane during development that prevents the insertion of the C5b–C9 complex into the outer membrane of the parasite (Sacks & Sher, 2002). Another mechanism of inhibition of complement by *Leishmania* parasites is through the expression of protein kinases that phosphorylate C3, C5 and C9. Finally, gp63 and LPG enable binding of C3bi to the parasite surface. Complement activation results in opsonization-binding of C3bi to the surface of the parasite, a process which is used by *Leishmania* parasites to escape from the hostile environment by phagocytosis via complement receptors (CR) (Dominguez *et al*., 2003; Olivier *et al*., 2005).

1.6.3.2 B cell immune response to *Leishmania* infection

Interaction between B and T lymphocytes results in signalling which is crucial in shaping the helper T cell responses (Rolf *et al*., 2010). During *Leishmania* infection, resistance depends on the generation of a protective Th1 response, whereas susceptibility is mediated with the generation of a Th2 response. Attempts were made to determine the lymphocyte of B cells for the development of T helper 1 and 2 responses during infection with *Leishmania major* (Fig 1.6.3.2). The progression of the disease and development of the T helper cell was assessed in mice infected with *L. major* and lacking B cells (by disruption of immunoglobulin M locus: mMT) on genetically susceptible Balb/c and resistant C57BL background. Interaction of CD40 and B7 (expressed on B cells) with CD40L and CD28 (expressed on T cells) respectively may be required for the acquisition of helper function (Liu *et al*., 1995; van Essen *et al*., 1995; Laurent *et al*., 2010). The importance of B cells was

investigated by antibody depletion or by T-cell reconstitution of SCID mice (Ronchese & Hausmann, 1993; von Stebu *et al*., 2004) or by genetic disruption of the immunoglobulin (Ig) locus (Chen *et al*., 1993; Kitamura *et al*., 1991).

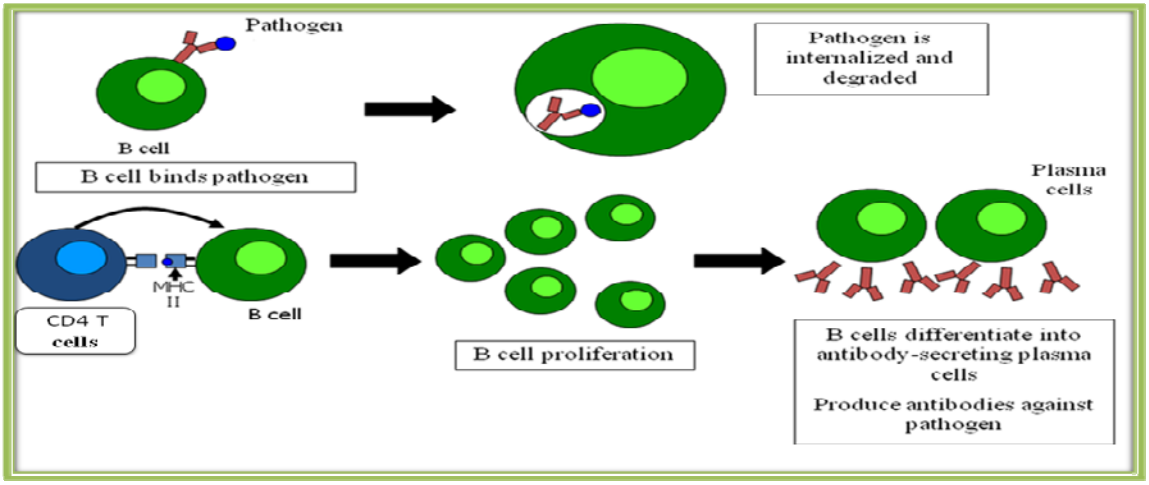


Figure 1.6.3.2 B cells immune response to *Leishmania* infection
The peptides from the pathogen are presented by MHC II to T cell resulting in the activation of the B cell and then B cells differentiate into antibody-secreting plasma cells, which produce antibodies against the pathogen (the digram is constructed based on information obtained from van Essen *et al*., 1995).

In *L. major* infections in the most resistant mouse strains produced a Th1 response and are therefore resistant. However, Balb/c mice showed an increased Th2 response, which is unable to restrain the development of the parasite (Reiner & Locksley, 1995; Radwanska *et al*., 2007). If B cells are essential for the production of a Th2 response during *L. major* infection, then Balb/c mice lacking B cells may be able to mount a protective Th1 response by default. In fact, Balb/c mice treated with anti-IgM are resistant to *L. major* and Balb/c X-linked immune-deficient (*Xid*) mice, which lack the B1 subset of B cells, showed improved resistance to *L. major* (Sacks *et al*., & Sher, 1984; von Stebu *et al*., 2004).

1.6.3.2.1 Antibody

Sera from patients with either mucosal or visceral manifestations of *Leishmania* infection were examined to determine the antibody class against the causative parasite by ELISA assay using intact promastigotes as antigen. Sera from all examined patients had significant levels of IgG and little or no IgA or IgM anti-parasite antibody. Antibody in the diagnosis of *Leishmania* infections has mainly relied on the detection of total immunoglobulins or IgG specific antibodies (Ho *et al*., 1983; Azmi *et al*., 2009). Sera from patients with confirmed mucosal or the visceral Leishmaniasis was examined to determine the classes and subclasses of Ig containing activity specific for the parasite. In a study by Mengistu *et al*., (1992) the classes of anti-*Leishmania* antibodies were determined in serum from patients with cutaneous Leishmaniasis using immunoblotting; differences in the pattern of antigen recognition by IgM, IgG, IgE and IgA antibodies were detected in DCL and LCL patient serum. All antibody classes demonstrated different patterns of banding to a variety of molecular species of antigens of the parasites. There was a significant difference in the specificity and reactivity of the IgG antibody among different individual patients. The IgM binding patterns were usually homogeneous and limited to antigens with M_r >40 kDa. IgA antibodies were the only class of antibodies (Anti-*Leishmania*) which showed a shared pattern of common antigen recognition in all the tested patients. However, the target antigens for IgE reactivity included two antigens of M_r 36 and 46-48 kDa which were not documented for other isotypes. Such antibody class reactivity may be useful in the design of serodiagnostic assays for the recognition of *Leishmania* infection (Bhowmick *et al*., 2009).

Antibodies play a number of functional roles in the pathogenesis of Leishmaniasis in humans. IgG serum antibodies reactive to *L. aethiopica* are observed in high titres in patients with diffuse cutaneous Leishmaniasis (DCL) and localized cutaneous Leishmaniasis (LCL) (Goto *et al*., 2010). At the level of antigen specific recognition the western blot profile of serum antibodies of any given DCL or LCL patient were limited to only a subset of the potential antigenic components expressed by the parasite and significant heterogeneity in the antibody repertoire of individual DCL and LCL patients was observed. It was also shown that the general distribution pattern and specificity of the classes of serum antibody developed in patients with cutaneous Leishmaniasis (Mengistu *et al*., 1990; Goto *et al*., 2010).

1.7 Immune evasion by *Leishmania*

In macrophages, intracellular *Leishmania* parasites require a number of immune evasion mechanisms to resist phagolysosome fusion and prevent activation of more-potent acquired immune responses. The main adaptive immune evasion strategies by the parasite include the inhibition of interleukin IL-12 synthesis, and induction of IL-10 and transforming growth factor-β (TGF-β) by infected cells. These cytokines promote the shift of response from T helper Th1 to Th2 that characterizes susceptibility to *Leishmania in vivo* (Taylor-Robinson, 2001; Ritter *et al*., 2009). Prior to internalisation, *Leishmania* promastigotes bind to surface molecules such as complement receptors 1 and 3 expressed by macrophages. The major macrophage ligand for mature promastigotes is CR1, however other parasite surface glycoproteins e.g. gp63 membrane protease, and additional macrophage receptors e.g. CR3, have been involved in the transformation of promastigotes into intracellular amastigotes (Moreno *et al*., 2010).

LPG and gp63 are responsible for the virulence of the parasite. In fact, LPG has been shown to be involved in survival within the insect and establishment of infection in the macrophage (Descoteaux & Turco, 1999). The roles of the LPG and gp63 pathogen-associated molecular patterns appear to complement each other, and these molecules appear to be the trigger of the immune response against *Leishmania* (Zambrano-Villa *et al*., 2002; Tuon *et al*., 2008). Some studies have demonstrated that LPG might inhibit the fusion of the phagosome with lysosomes, an essential step in the destruction of the pathogen. And, vacuoles formed around a *Leishmania* mutant lacking the cell surface LPG fused extensively with endosomes and lysosomes, promoting complete destruction of the parasite (Dermine *et al*., 2000; Desjardins & Descoteaux, 1997; Tuon *et al*., 2008). gp63 has been shown to inhibit some degradative phagolysosomal enzymes (Sorensen *et al*., 1994) and although both antigens (LPG and gp63) may be considered important inhibitors of macrophage activation, other factors are also involved including (i) alterations in the cyclooxygenase and lipooxygenase pathways, (ii) suppression of macrophage expression of class I and class II MHC gene products, (iii) defective regulation of calcium-dependent signalling, (iv) altered activation and translocation of protein kinase C, and (v) activation of the Src homology 2 domain containing tyrosine phosphatase-1 (Kwan *et al*., 1992; Olivier *et al*., 1992; Mukhopadhyay *et al*., 2006). Thus, it could be concluded that complex mechanisms are involved in amastigote survival in the macrophage and those are not only related to external proteins and it is clear that GPIL, LPG, and gp63 are considered to be the first antigens to encounter the innate immune response (Nandan *et al*., 2002; Tuon *et al*., 2008).

It is documented that the presence of *Leishmania* in a tissue encourages the production of cytokines by APCs. IL-12 is one of the main cytokine responsible for

the principal Th1 response and the achievement of the adaptive cellular response in individuals with Leishmaniasis (Chatelain *et al*., 1992; Tuon *et al*., 2007; Berberich *et al*., 2003).

Mbow *et al*., (2001) have shown that the expression of co-stimulatory molecules influence the product of antigen specific T cell priming. It was found that *L. major* effected the expression of co-stimulatory molecules on different populations of epidermal cells. CD86 expression was down-regulated on Th1 epidermal cells (keratinocytes) from resistant C3H mice, but not from susceptible Balb/c mice. In addition, epidermal cells from Balb/c mice showed a down-regulation of CD80 expression on DC Langerhans cells. *In vitro* T cell priming experiments, using syngeneic epidermal cells as antigen-presenting cells (APC), showed that the production of IFN-γ was inhibited when either CD80 or CD86 signalling pathways were blocked. In addition up-regulation of CD80 on *Leishmania* antigen-treated human macrophages and DCs has been shown. Also, human responses to *L. major* are dependent on CD40 co-stimulation and influenced by both CD80 and CD86 expression (Brodskyn *et al*., 2001; Goronzy & Weyan, 2008). Moreover, *Leishmania* down regulates the production of antigen specific CD4+ T helper cells which produce IFN-γ and IL-12, essential for the activation of the macrophages to kill the intracellular amastigotes. *Leishmania* infection was shown to induce the production of TGF-β and IL-10 which prevents the killing of intracellular amastigotes (Bogdan *et al*., 1996; Bhowmick et al., 2009).

1.8 Vaccination Strategies

The clinical manifestations of human Leishmaniasis depend on the parasite species and on the status of the host immunity. Although chemotherapies against

Leishmaniasis do exist, they are few and expensive, particularly for people in developing countries. Side effects and drug resistance are also adverse results of chemotherapy. Considerable efforts have been devoted to the development of vaccines against *Leishmania* infection and crude or purified antigens have been shown to elicit a certain degree of protection in susceptible mouse strains such as Balb/c mice. Vaccination has been proposed to be the most cost-effective measure to control Leishmaniasis.

1.8.1 Leishmanisation

Resistance to *Leishmania* disease is mediated by antigen specific T cells which give life-long immunity against reinfection with the same species. It is known that deliberate infection of infants with exudates of cutaneous *Leishmania* lesion on the buttocks prevented subsequent lesions on the face. This process was called 'Leishmanisation'. It is generally known in the hyper-endemic Asian countries that after recovery from CL, individuals are usually protected against further lesions (Coler & Reed, 2005; Nylen & Gautam 2010). Currently, the improvement of a new vaccine must meet several strict criteria where safety is the corner stone in every vaccine licensed for clinical use. 'Leishmanisation', the oldest form of vaccination against cutaneous Leishmaniasis, has been practiced for centuries in the Middle East and Russia (Handman, 2001; Ali *et al*., 2009) and where recovery from CL is followed by long lasting immunity to the disease (Khamesipour *et al*., 2006). Live virulent *L. major* promastigotes were harvested and used in large-scale vaccination trials during the 1970s and 1980s in Iran and the Soviet Union (Handman, 2001; Khamesipour *et al*., 2005). Though still practiced in Uzbekistan, the noteable side effects, including the progress of large constant lesions, psoriasis and

immunosuppression, led to the discontinuation of Leishmanisation in many countries and the focus of vaccine development consequently shifted towards the use of killed organisms. The possibility of using Leishmanisation as a live challenge for the evaluation of Leishmanisation vaccines was first considered by the WHO in 1984 during the large scale Leishmanisation program in Iran (Khamesipour *et al*., 2005). Although Leishmanisation gave a high percentage of successful lesion development and subsequent immunity to infection with *L. major*, it was neither reproducible nor safe (Handman, 2001; Ameen, 2009) and is not recommended by the WHO. Live-non-attenuated vaccines have been experimentally tested using non-pathogenic species, such as *Leishmania tarentolae,* based on the notion that high levels of immunological cross-reactivity between species at both the humoral and cellular level could provide potent cross immunity against virulent species. Cross immunisation is a promising approach to vaccination against visceral Leishmaniasis caused by *L. donovani* since Balb/c mice were able to mount a protective immune response after only a single peritoneal vaccination with a live *Leishmania tarentolae* (Breton *et al*., 2005; Rafati *et al*., 2011).

1.8.2 Live-Attenuated Vaccines

The genetic modification of *Leishmania* parasites to reduce virulence without reducing immunogenicity is of current interest in the development of a *Leishmania* vaccine. It is an interesting approach, as attenuated parasites closely mimic natural infection that may well lead to similar immune responses without the risks associated with infection with virulent live *Leishmania* parasites (Handman, 2001, Abdus Salam, 2009). Due to advances in molecular biology and the genomic sequencing of *L. major,* the attenuation of *Leishmania* parasites by blocking, removing or replacing

essential genes became possible (Coler & Reed, 2005; Roberts, 2005). *Leishmania* can be engineered for safe immunisation by knocking out the genes required for a long term survival in the host and the deletion of the dihydrofolate Reductase Thymidylate Synthase gene (DHFR-TS) in *L. major* has produced the first *Leishmania* 'knockout' vaccine tested. This gave significant, but temporary, protection in mice when challenged with the wild type organism and also gave unsatisfactory results during additional studies in monkeys (Khamesipour *et al*., 2006). The production of *Leishmania* 'knockouts' is not only able to identify virulence genes as new potential target antigens, but can also lead to a better understanding of the biology of the parasite. Additionally, studies using knockout parasites may be key in identifying specific genes for the development of DNA vaccines as shown in the study by Selvapandiyan *et al*., (2007) have demonstrated the importance of the centrin proteins for the duplication and progression of the *Leishmania* amastigote cell cycle. Palatnik-de-Sousa, (2008) constructed a DNA vaccine cassette suitable for Leishmanisation as an efficient treatment of non resolving lesions. On the other hand, parasites can be modified to produce biological substances that generate immune attack, such as granulocyte monocyte colony stimulating factor (GM-CSF) (Dumas *et al*., 2003). The attenuated vaccines of *Leishmania* parasites offer a novel approach to immunisation against Leishmaniasis, nevertheless there are concerns that the parasite may revert to a virulent form. In addition, targeted deletion of essential virulence genes can result in complete damage of the parasite or production of mutants that only delay development of lesion (Breton *et al*., 2005).

1.8.3 Killed *Leishmania* parasites

Killed *Leishmania* parasites are interesting for their stability, in terms of biochemical composition and antigenicity, and also for low cost and safety (Giunchetti *et al*., 2008). Killed *Leishmania* parasites have been used for vaccine studies, with or without adjuvants. Autoclaved *Leishmania* promastigotes, as an example, with or without BCG (*Bacillus* of Calmette and Guerin) as adjuvant have been tested against visceral and cutaneous Leishmaniasis in a randomized, BCG-controlled clinical trial. It was found that two injections of the vaccine autoclaved *L. major* (ALM) with BCG were significantly better than BCG alone (Khalil *et al*., 2000; Palatnik-de-Sousa, 2008). Most of the studies in America using killed *Leishmania* vaccine have used autoclaved *L. amazonensis* lysates in some instances compared with native species, while in most of the studies on vaccines against Old World Leishmaniasis *L. major* has been used. In the Middle East killed *Leishmania* vaccines failed to give significant protection against Leishmaniasis in humans (Handman, 2001). Also, killed *Leishmania* vaccines tested in humans and dogs, in Asia and South America since 1940 induce low efficacies and poor protection in vaccinated subjects (Palatnik-de-Sousa, 2008). However, studies using killed *Leishmania* vaccine in mouse models have demonstrated that the injection of vaccines intravenously or intraperitoneally, but not subcutaneously, was associated with excellent protection, and subsequently several formulations of killed vaccines have been developed. Those studies emphasise that the site of administration influences the efficacy of a vaccine therefore; In Venezuela, autoclaved *L. mexicana* is currently used for immunotherapy to treat patients with cutaneous Leishmaniasis (Khamesipour *et al*., 2006).

1.8.4 Recombinant Protein Vaccines

Recombinant protein vaccines are produced from the cells engineered genetically to express foreign genes encoding antigenic proteins (Khamesipour *et al*., 2006). Different *Leishmania* recombinant proteins have been tested for their potential as candidate vaccines, such as recombinant hydrophilic acylated surface protein B1 (HASPB1) which induced protection against challenge with *L. donovani* in a mouse model (Stager *et al*., 2000). The recognition of defined parasite peptides and proteins that promote useful immune responses may contribute to vaccine progress. gp63, the major *Leishmania* surface glycoprotein, is highly conserved across species (Medina-Acosta *et al*., 1993, Knox *et al*., 2010). LACK (*Leishmania* analogue of the receptors of activated C kinase) antigen, in spite of stimulating a strong Th2 response in infected mice, induces substantial protection in Balb/c mice if administered in conjunction with adjuvants that stimulate a Th1 response. Thus during vaccine development, immunogenicity and the amount of antigen expressed by parasite *in vivo* are two important factors which need to be considered along with the necessity for adjuvants to elicit a strong Th1 response. In another recent study, the *Leishmania* elongation initiation factor (LeIF) has been considered as a vaccine candidate based on its ability to induce Th1-type cytokines in humans (Coler & Reed, 2005). A *Leishmania* vaccine containing Fucose-mannose ligand (FML) has been tested to prevent canine visceral Leishmaniasis in an endemic area in Brazil. This study evaluated the immune response of dogs vaccinated (20 vaccinated and 20 controls) with FML against total antigen of *Leishmania chagasi* (TAg) and FML alone. The vaccine was given 3 times S.C. at 21 day intervals. The proliferation responses and antibody production against FML or total promastigote antigen were determined in the PBMCs that have been isolated before and 10 days after vaccination. The vaccine

induced humoral responses in 100% of the tested animals against both antigens but less cellular immunity to FML (85%) and total antigen (80%). The supernatants of cultured cells stimulated with TAg and FML showed an increase in IFN-γ. Reduced numbers of CD4+CD25+ T cells were detected in the vaccinated group compared to that observed before vaccination (Lima *et al.,* 2010).

1.8.5 DNA vaccines

DNA vaccines represent a relatively simple formulation for both therapeutic and prophylactic purposes, containing the gene encoding the antigen which can stimulate the immune system, upon gene expression *in vivo*. The DNA vaccines have now entered clinical trials, targeted to prevent and cure many infectious disorders, cancer, autoimmune disease and allergic diseases. DNA vaccines are also widely used in proteomic research to understand immunological phenomena, such as antigen presentation and cross priming (Donnelly *et al*., 2005; Montalvo-Alvarez *et al*., 2008). Cross priming is a process where extracellular antigens are processed by APC and transported from endosomes to cystoles to be presented on MHC I and MHC II to CD8 and CD4 T lymphocytes respectively. Transfected DCs with DNA vaccine could activate CD8+ and CD4+ T cells via MHC class I and MHC class II respectively against the DNA encoded antigen. Soluble proteins and fragments from apoptotic transfected cells can also be endocytosed by immature DC to be expressed on MHC class I or MHC class II, leading to their differentiation into mature DCs. Thus, a DNA vaccine can be effective in the stimulation of both CD8+ and CD4+ T cell populations (Ali *et al*., 2009; Vanloubbeeck and Jones 2004). Therefore, cross presentation is an essential mechanism for the generation of CTL responses to antigens (Han *et al.,* 2005). The history of DNA vaccine development dates back to

1990, when Flenger and colleagues showed that a simple bacterial plasmid vehicle containing the gene for a marker protein along with a promoter can be functional in a mammalian model (Wolff & Budker, 2005). They observed that the intramuscular (I.M) injection of formulation containing a gene encoding for a viral protein can trigger the generation of the CD8+ cells, CTLs and antibodies in mice. They also found that the CTLs were able to protect mice from viral challenge (Donnelly *et al*., 1997; Kashyap *et al*., 2010). DNA vaccines allow protein expression when DNA constructs encoding appropriate protective antigens are injected into mammalian cells and they have the capacity to strongly induce both humoral and cell mediated immunity. The immunogenicity can be increased by manipulating the vector or by incorporating a cytokine gene which serves as an adjuvant (Ivory & Chadee, 2004). DNA vaccines can be administered intradermally, intramuscularly and even intranasally (Mendez *et al*., 2002; Tesoro-Cruz *et al*., 2008). DNA vaccines encoding some of the *Leishmania* antigens have been investigated by several groups, based on the introduction of a plasmid DNA encoding the gene for an antigenic protein into host cells *in vivo*. The endogenous expression of a foreign antigen may induce strong antibody production as well as a complete cell-mediated immune response, because DNA vaccines have a strong bias to Th1 response. It has been reported that CPG-DNA sequences act as adjuvant and stimulate humoral and cellular immunity and promote Th1 differentiation in aged Balb/c mice (Maletto *et al*., 2002).

Recently, it has been shown that DNA vaccination induced strong immunoprotection against cutaneous and visceral Leishmaniasis (Carrion *et al*., 2008) potent anti-*Leishmania* immune responses in mice, which in these models a combination of multiple antigen encoding plasmids have improved protection (Iborra *et al*., 2004). Introduction of DNA into monocytes, macrophages and dendritic cells was shown to

increase their antigen uptake and presentation, as well as augment the expression of co-stimulatory molecules and the secretion of pro inflammatory cytokines such as IL-10 and IL-12. These cytokines in turn activate NK cells, CD4+ and CD8+ T cells, enhancing their lytic activity and the secretion of high levels of IFN-γ (Krieg, 2002). In addition, Balb/c mice immunised with a DNA vaccine encoding the nucleosomal histones from *L. infantum* induced significant protection against VL and dogs vaccinated by a prime-boost regime with DNA-LACK followed by an Ankara virus triggered a Th1 type immune response, leading to protection against canine VL (Carrion *et al*., 2008; Ramos *et al*., 2008). This protection correlated with the absence of VL symptoms, lower *Leishmania*-specific antibodies, and a higher degree of T cell activation in *Leishmania*-target organs co-incidental with a higher synthesis of Th1 cytokines (Dumonteil *et al*., 2003; Murray *et al*., 2005). These generate protective responses against Leishmaniasis and represent a promising approach to vaccine development (Encke *et al*., 1999; Mendez *et al*., 2002). DNA vaccines are advantageous over other vaccine strategies and several features have made them a promising alternative (Donnelly *et al*., 2000). They are easily produced, simple and can be cheaply produced on a large scale; because of their temperature stability they can be easily stored and transported.

A single plasmid encoding several antigens and multiple plasmids encoding different antigens, can be delivered in a single administration, thus making DNA vaccines extremely flexible (Encke *et al*., 1999) thus providing resistance against more than one species. Furthermore, bacteria derived DNA plasmids are naturally immunogenic as their backbones contain unmethylated cytosine phosphate guanosine (CpG) motifs which have been shown to readily induce Th1 cytokine expression and increase CD8 T cell responses (Garmory *et al*., 2004). This adjuvant property is of great value,

ensuring the induction of cell-mediated immunity and in turn conferring protection against the parasite.

1.8.5.1 Administration of DNA Vaccines

Several methods of DNA vaccine administration have been tested and it is thought that the route and site of immunisation plays a critical part in influencing the nature of the immune response elicited. To date, successful DNA vaccination has been administered through intramuscular, intravenous, intraepidermal, intraperitoneal, intravaginal, intranasal, intrasplenic, intrahepatic, subcutaneous or oral routes (Handman *et al*., 2000; Ali *et al*., 2009). Of these intramuscular administration is the most popular (Garmory *et al*., 2003; Mendez *et al*., 2002). The dose of DNA vaccine necessary for protection in C57BL/6 mice against *Leishmania major* was 5 times smaller when delivered by gene gun (particle-mediated epidermal delivery) than by either intramuscular or subcutaneous injection. Furthermore, Ali *et al*., (2009) found that 1µg of DNA encoding *L. mexicana* gp63, administered using gene gun, conferred better protection in susceptible Balb/c mice than 100µg of vaccine administered intramuscularly. These findings clearly demonstrate that the efficacy of a DNA vaccine is greater using gene gun administration than intramuscular injection. This may be due to the fact that gene gun administration can directly transfect APC, such as DC, with the plasmid DNA (Encke *et al*., 1999). In a previous study it was shown that, protection of mice against challenge with *L. major* was dependent on the frequency of immunisation with killed parasites either mixed with rIL-12 or alone with highest protection in mice immunised 5 times per week. However, no protection was obtained from mice immunised with a single or double immunisation of killed parasites either alone or with repeated rIL-12 inoculation (Okwor *et al.*, 2010).

Flying vaccinator is a novel idea of using genetically engineered hematophagous insects to deliver vaccines has been recently reported by Yamamoto *et al*., (2010). A transgenic anopheline mosquito that expressed the *Leishmania* vaccine candidate, SP15, fused to monomeric red fluorescent protein (mDsRed) in its salivary glands have significantly induced anti-SP15 in bitten mice demonstrating the ability of delivering antigens through blood feeding. Thus, this technology makes the generation of transgenic mosquitoes possible to match the original idea of a flying vaccinator. This has shown that the field of DNA vaccines is evolving to adopt novel technologies and delivery techniques to significantly improve protective immune responses.

1.8.5.2 Immune mechanisms of DNA Vaccines

The gene gun delivers DNA-coated gold particles at high velocity directly into cells of the epidermis, which include skin cells, LC and dermal DCs. On entering the cells, the plasmid travels to the nucleus, where the transcription of the encoded gene into protein takes place, which is then processed into peptides by proteases and presented on MHC I which stimulates CD8 T lymphocytes (Fig 1.8.5.2) (Encke *et al*., 1999). DC directly transfected with the DNA vaccine can prime CD8 T cells by presenting the DNA encoded antigen in the context of MHC class I (Vanloubbeeck & Jones, 2004). On the other hand, there is evidence to suggest that immature DC can endocytose soluble proteins and fragments from apoptotic transfected cells and express the coded antigen through MHC class I or MHC class II after differentiating into mature DC (Donnelly *et al*., 2000). DNA vaccination thus can result in the stimulation of both CD4 T and CD8 T cell populations. DC, play a major role in the induction of both humoral and cell-mediated immunity following DNA vaccination

due to its cross priming ability (Vanloubbeeck & Jones, 2004). A number of studies have been conducted on potential DNA vaccines against *Leishmania* and according to results by Handman *et al*., (2000) such vaccines can be used therapeutically to treat cutaneous Leishmaniasis caused by *L. major* in both genetically susceptible Balb/c mice and resistant C3H/He mice.

There is clearly controversy regarding the nature of the antigens that induce protective immunity in different species.

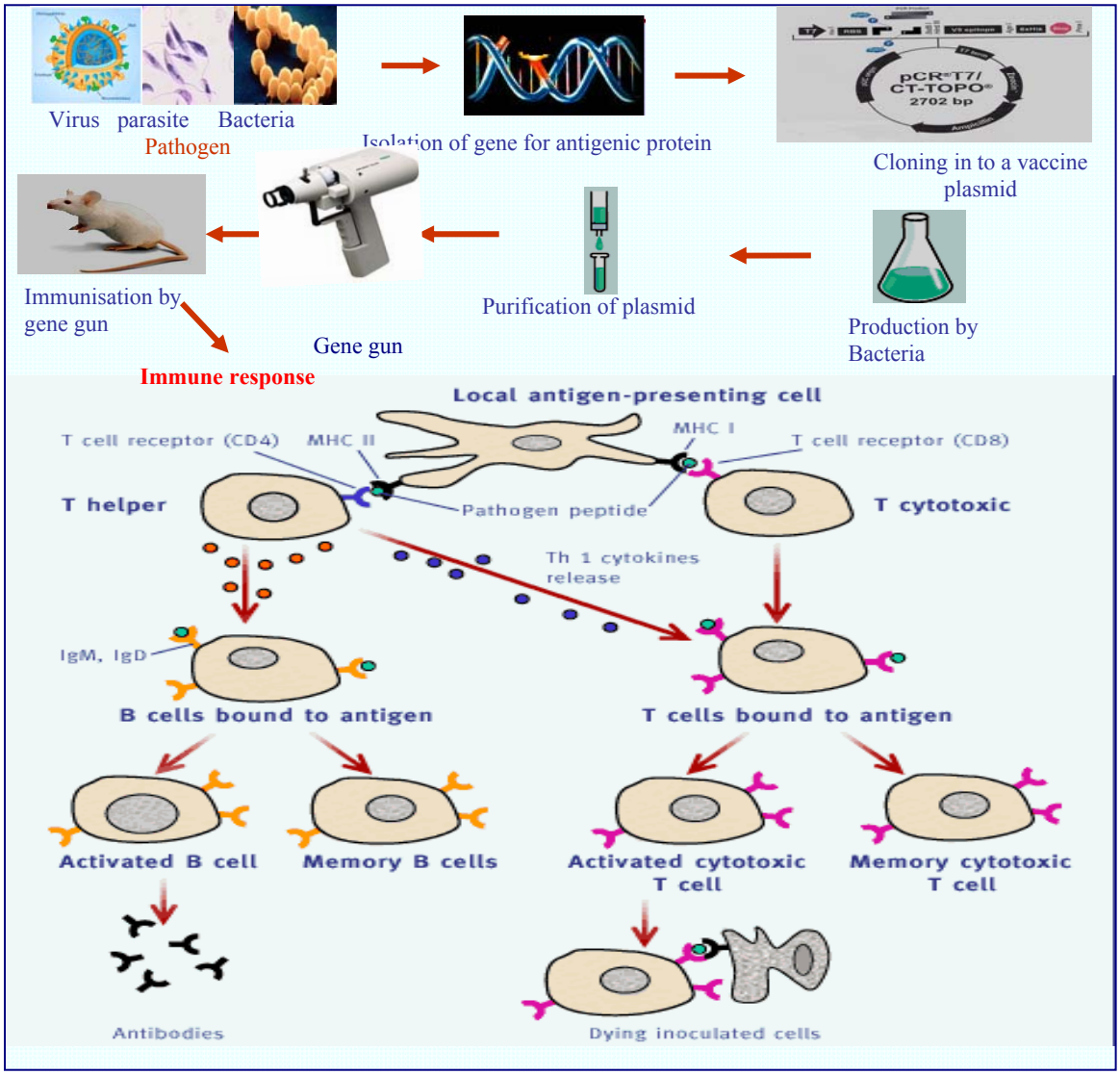


Figure 1.8.5.2 DNA vaccines
The figure shows, activation of $CD8^+$ T lymphocytes, implicated in host defence against intracellular pathogens via cytotoxic T lymphocytes (CTL), and $CD4^+$ T lymphocytes, which secrete cytokines and play a role in production of specific antibodies (Kowalczyk & Ertl, 1999).

9 Aims of study

The aim of this study is to develop an animal model (Balb/c mouse model) to study immunogenicity of SLA antigens fractionated by anion exchange ‘’Mono Q column’’ trying to identify immune dominant antigens. Immune responses will be measured by CTL assay, proliferation assay and cytokines measurement.

This study aimed to investigate the effect of live and autoclaved *L. mexicana* on the expression of cell surface markers including MHC class I, MHC class II, CD40, CD80 and CD11c in DCs and susceptibility to CTL killing. The reverse effect of fungizone on susceptibility to CTL killing and the expression of MHC I, MHC II, CD40, CD80 and CD11c in the DCs following *Leishmania* infection will also be investigated.

The discovery of new antigens is essential to identify and characterise antigens with a potential application as a novel vaccine candidate. The immunogenicity of centrin genes, newly identified *Leishmania* antigens, have not previously been studied and very little is known about on their biology in *Leishmania.* Hence, this study is also aimed at determining the immunogenicity of *Leishmania donovani centrin-3* (*Ldcen-3*). Two plasmid constructs containing *Ldcen-3* (pCRT7/CT-TOPO-*Ldcen-3* and pcDNA3.1/Hygro-*Ldcen-3)* will be used for immunisation of Balb/c mice by gene gun. Immunity will be measured by protection against challenge with live *L. mexicania* parasite and measuring CTL activity levels in immunised mice against DCs loaded with SLA and CT26 tumour cells transfected with pcDNA3.1 (-)-*Ldcen-3*.

Chapter 2 Methods

2 Methods

2.1 Preparation of Soluble *Leishmania* Antigen (SLA)

L. mexicana promastigotes 2×10^9 (strain Hd18 kindly provided by Dr. Varley, the London School of Hygiene and Tropical Medicine) were washed 4 times in PBS and resuspended in 3 ml of *Leishmania* buffer; 100mM Tris buffer, pH 7.3 containing 1mM EDTA, 0.5mM PMSF (Sigma) and 2.5mg/ml Leupeptin (Sigma). The parasites were lysed by sonication for 2 minutes and the lysate was centrifuged at 13000g for 20 minutes. The supernatant was further ultracentrifuged for 4 hours at 100,000g. The antigen produced by this procedure was called SLA1. Another antigen preparation, SLA2 was similarly produced but without ultracentrifugation. Both SLA1 and SLA2 were dialysed against 5 litres of cold PBS overnight with continuous agitation and several changes of the PBS. The dialysate was sterilised by passing through 0.2 μm filters (Sartorius), then kept at -80˚C.

2.2 Western Blotting to detect gp63 in SLA

2.2.1 Protein Assay

A protein assay was set up to measure the concentration of the unknown protein samples (mg/ml). The total protein in each antigen preparation was measured using the Sigma Bicinchoninic Acid Protein Assay Kit according to the manufacturers' instructions. The kit contains reagent A which is a 1,000 ml solution containing bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 N NaOH and reagent B which is a 25 ml solution containing copper (II) sulphate

pentahydrate. Bovine serum albumin (BSA) was used as standard protein. Briefly, 25µl per well of the antigen sample (SLA) and a serial dilution of 1mg/ml BSA in lysate buffer was used as standard and were placed in duplicate in 96-well plates (Biorad). Reagents A and B were mixed in the ratio of 50:1 and 200µl of the mixture was added per well. The plate was wrapped in foil and incubated at 37 ºC for 20-30 minutes to develop the reaction and the plate was then read at 570nm on a Spectrophotometer (Tecan). A standard curve was created to determine the protein concentration of each unknown sample by Excel Microsoft.

2.2.2 Western Blotting

The gel tank was assembled according to the instructions and 10% a resolving gel: 1165µl acrylamide, (30%) 875µl Tris 1.5 M HCl pH 8.8, 1460µl H_2O, 35µl ammonium persulphate 10%, 3.5µl Tetramethylethylenediamine (TEMED), was prepared and poured into the cassette. The gel was left until it solidified. The 4% stacking gel (15% acrylamide /bis, 25% 0.5 M Tris HCL, pH6.8, 60% dH_20 plus 0.1% TEMED and 10% ammonium persulphate) was added on top of the resolving gel and the comb was inserted in it. To prepare the samples, 33µl of 1x reducing sample buffer (Dithiothreitol DTT is a reducing agent used to disrupt disulphide bonds to ensure the protein is fully denatured before loading on the gel) was mixed with a 100µg of each sample (SLA2 and SLA2 fractions) and heated to 95˚C for 5 minutes and then 20µl of each sample was loaded into the gel. Ten to thirty µg of samples was run at 90V through the stacking gel and 120V through the resolving gel. Standard protein ladder (10-200 kDa) (Invitrogen) was used to assess the molecular weight of the sample proteins. After running the samples throughout the gels, the resolving gel was incubated in transfer buffer (48mM tris, 39mM glycine, 200ml

methanol, 800ml water, pH 9.2) for 5 minutes and then proteins were transferred onto the Bio-trace membrane (nitrocellulose membrane) at 13V for 30-40 minutes through a semi-dry transfer system using trans-blot machine (Biorad) according to manufacturers' instructions. To detect *Leishmania* gp63 protein in SLA preparations, the membrane prepared from SDS-PAGE membrane was blocked overnight in TBS + 0.05% Tween 20 (TBS-T) + 5% Marvel milk powder at 4°C under constant agitation. The primary rabbit anti *L. mexicana* gp63 antibody (Gift from Dr. McGwire, The Ohio State University) was then added at 1:1000 dilution in TBS + 0.05% Tween 20 + 5% Marvel milk powder and incubated for 1 hr at room temperature with vigorous shaking. After washing the membrane 3 times for 15 minutes in TBS-T at room temperature, the secondary antibody (HRP conjugated goat anti rabbit IgG (Biorad) was added to the membrane at a 1: 2000 dilution in 5% milk-TBS-T and incubated for 1 hour at room temperature with vigorous shaking. The membrane was then washed 4 times for 15 minutes at room temperature in TBS-T, and detected using ECL kit (Amersham). Briefly, equal parts of solutions A and B were mixed and incubated for 1 minute. A cellulose membrane was placed down on saran wrap, and the bubbles were completely removed. The membrane was fixed by tape to the inside of the film cassette in the dark and exposed to a sheet of x-ray film for up to 45 minutes before developing the band to the film.

2.3 *L. mexicana* promastigotes culture

L. mexicana promastigote strain Hd18 was cultured in Schneider media (Sigma) supplemented with 10% FCS at 25 ºC as described by Bates (1994). *Leishmania mexicana* promastigotes were cultured, starting with 1×10^6 of parasites in 10ml media in T25TC flasks, and were counted every two or three days. 10µl of the parasites culture was diluted twice in 90µl 1% of paraformaldehyde and counted at 1×10^6/ml.

2.4 Cytotoxic T-Lymphocyte Assay

2.4.1 Generation of splenocytes

Between 2-3 days prior to the removal of spleens from immunised mice, naïve splenocytes were cultured at 1.5×10^6 cells/ml in 40ml T cell media (RPMI 1640 supplemented with 1% L-glutamine, 10% FCS, 20mM HEPES buffer, 50μM 2-mercaptoethanol, 50U/ml penicillin, 50μg streptomycin and 0.25μg/ml fungizone) containing 25μg/ml LPS the outer membrane of Gram-negative bacteria (Sigma) and 7μg/ml dextran sulphate in a T75 culture flask and incubated at 37°C in a 5% CO_2 atmosphere. Naïve splenocytes treated with LPS were irradiated at 3000 rads for 4 minutes. Cells were washed and pulsed with 100μg/ml SLA for at least 1 hour. Cells were then washed, counted and added to culture plates containing splenocytes from immunised mice at 5×10^5/well.

2.4.2 Generation of BM-DCs

Balb/c mouse BM-DCs were generated as described by Inaba with slight modification (Inaba *et al*., 1992). Bone-marrow cells were flushed out with media and harvested. (About 20×10^6 BMDCs were usually obtained from each mouse). Cells were then centrifuged and resuspended in 1ml BM-DC media, counted and plated at 1×10^6 cells per ml in T75 flask or 24 well plates with 100ng/ml of mGM-CSF and incubated at 37° C, 5% CO_2 atmosphere. On day 2 and day 4, non-adherent cells were washed out by gently replacing 75% of media with fresh DC media containing GM-CSF. On day 6, BM-DC was split into two groups. The first group (test) was pulsed with 10μg/ml SLA or 10 autoclaved parasites per 1 DC and the

second group was used as control. In some experiments, adherent cells were collected and treated in the similar way for phenotypic analysis. Control and test groups were incubated for 24 hours with 1μg/ml LPS to induce maturation. The following day, BM-DCs were washed in serum free RPMI 1640 media, counted and injected intradermally at 2×10^6 per mouse or used as target cells in standard 4-hour cytotoxicity assay.

2.4.3 *In vitro* generation of CTLs

Spleens were harvested from immunised and naïve mice and prepared in sterile conditions. Cells were flushed out from the spleens with serum-free RPMI 1640 media using a 25-G needle and 10 ml syringe. The spleen tissue was disrupted by pipetting and the cells were collected, washed and resuspended in CTL media. The cells were counted and plated in a 24 well plate at 2.5×10^6 cells/500μl/well. 5×10^5/500μl irradiated and SLA pulsed LPS blasts were then added and cultured for up to 5 days at 37°C in 5% CO_2. Supernatants were collected usually on day 3 and 5 for cytokine testing.

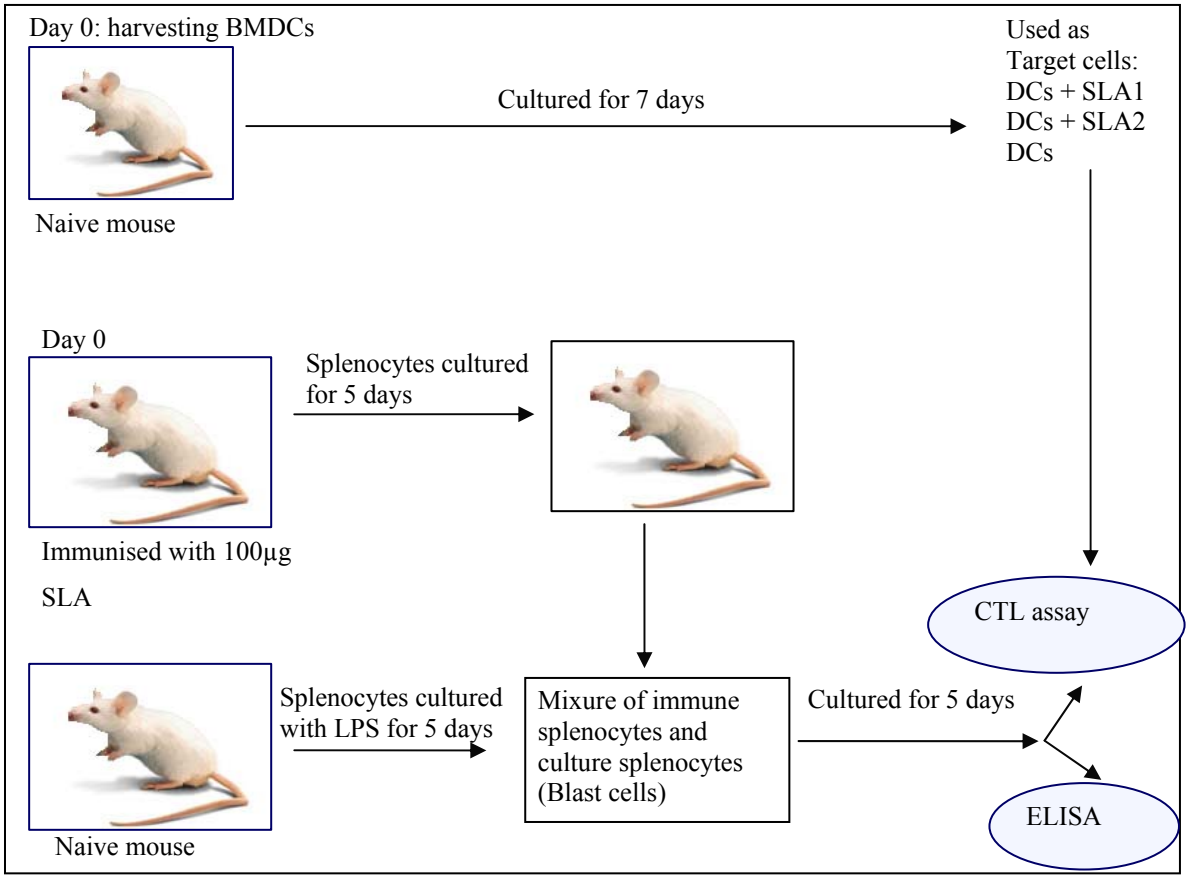


Figure 2.4.3 Protocol to generate CTLs from Balb/c mice immunised with SLA and DCs+SLA spleens harvested from immunised and naïve mice and splenocytes were collected as described in 2.4.2 and 2.4.3.

2.4.4 A radioactive Standard 4-hour Chromium Release Cytotoxicity Assay

On day 5 of the *in vitro* stimulation, splenocytes were harvested, washed twice in serum free medium, counted and resuspended in CTL media (see materials in appendix) and used as effector cells. Target cells (DCs or transfected tumour cells) were also harvested, washed and labelled at a concentration of 1×10^6cells/ml with 100µl Ci of chromium-51 (Amersham,UK) followed by 1h incubation at 37°C. The labelled cells were then washed and suspended in medium and pulsed with SLA (as DCs targets) and incubated for 1 hour at 37°C. Effector and target cells were mixed in a volume of 200µl in 96 well plates, at ratios of (E: 5×10^5 to T: 5×10^3 (100:1), E: 25×10^4 to T: 5×10^3 (50:1), E: 152×10^3 to 5×10^3 (25:1), E: 625×10^2 to T: 5×10^3 (12:1)

and E: 31125×10^{1} to T: 5×10^{3} (1:6) in 200 µl medium and incubated for 4 hours at 37°C. Maximum ^{51}Cr release was determined by adding 50µl of 0.1% SDS, and spontaneous ^{51}Cr release was determined in the wells that contained target cells and medium only. Assays were performed in triplicate. The radioactivity of the released ^{51}Cr (the specific cytotoxicity) was determined using the following formulae; E: effector cells, T: target cells.

$$\text{percentage cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

2.5 Antibody/Cytokine Response

2.5.1 Detection of anti-*Leishmania* IgG1 and IgG2a isotype antibodies

Immunised mice were bled 4 times at weekly intervals started one week after immunisation. The blood samples were harvested in clean Eppendorf tubes and centrifuged at 200g for 10 minutes. The serum was collected and stored at -20ºC until tested for specific immunoglobulin by ELISA. Serum samples from naïve mice were also collected as controls. *L. mexicana* Soluble Antigen (SLA) 1µg/well was coated onto the flat bottom 96-well plates (Biorad) and incubated overnight at room temp. After 4 washes with PBS, 1:100 dilution of the serum samples in dilution reagent (1% BSA, 0.05% Tween 20 in 20mM Trizma base, 150mM NaCl, pH 7.2-7.4) was added in duplicate followed by 2h incubation at room temperature and 4 washes with PBS. The plates were blocked with blocking buffer (1% BSA, 5% sucrose in PBS with 0.05 NaN_3) for 1hour, then washed 4 times with PBS. Rabbit anti-mouse IgG1 and IgG2a (Serotec) were added separately for 1 hour, followed by goat anti-rabbit

antibody-HRP conjugated at 1:1000 dilution. The plates were stored at room temp for 1h followed by 4 washes. 50µl of Streptavidin-HRP; (HRP is a horseradish peroxidase enzyme) (DAKO) were added and the plate was kept at room temp for 20 minutes. 50µl/well from a mixture of equal volumes of A&B reagents were added to the plates for 20 minutes. 50µl/well of 2.5M H_2SO_4 was added to stop the reaction and the OD was measured at 450 nm by spectrophotometer.

2.5.2 Cytokine Assays (IFN-γ, IL-2, IL-4 & IL-12)

Supernatants were collected from splenocyte cultures during proliferation assays and stored at -20ºC until required. Cytokine analysis for IFN-γ, IL-2, IL-4 & IL-12 using ELISA kits (R&D Systems, Abingdon, UK) was performed according to the manufacturers' instructions (Fig 2.5.2 and Table 2.5.1). Briefly 96 wells/plates were coated with capture antibody 100µl/well and incubated overnight at room temperature, then washed three times with wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4). The plate was then blocked with 100µl/well of block buffer (1% BSA in PBS with 0.05% NaN_3) and then incubated for 1 hour at room temperature, then washed two times. And then 100 µl/well of sample or standard in reagent diluent (1% BSA, 0.05% Tween 20 in 20mM Tris, 150 mM NaCl), pH 7.2-7.4 was added and incubated for 2 hours at room temperature, and then washed two times. 100µl/well of detection antibody diluted in reagent diluent was added and incubated for 2 hours at room temperature, washed two times and then 100 µl/well of working dilution of Streptavidin-HRP was added and incubated for 20 minutes at room temperature, and then washed two times. 100µl/well of substrate solution (A&B reagents) was added and incubated for 20 minutes at room temperature followed by the addition of 50µl/well of stop solution and the optical density was immediately determined at 450

nm by spectrophotometer. A standard curve was created to determine the level of cytokine (Fig 2.5.2).

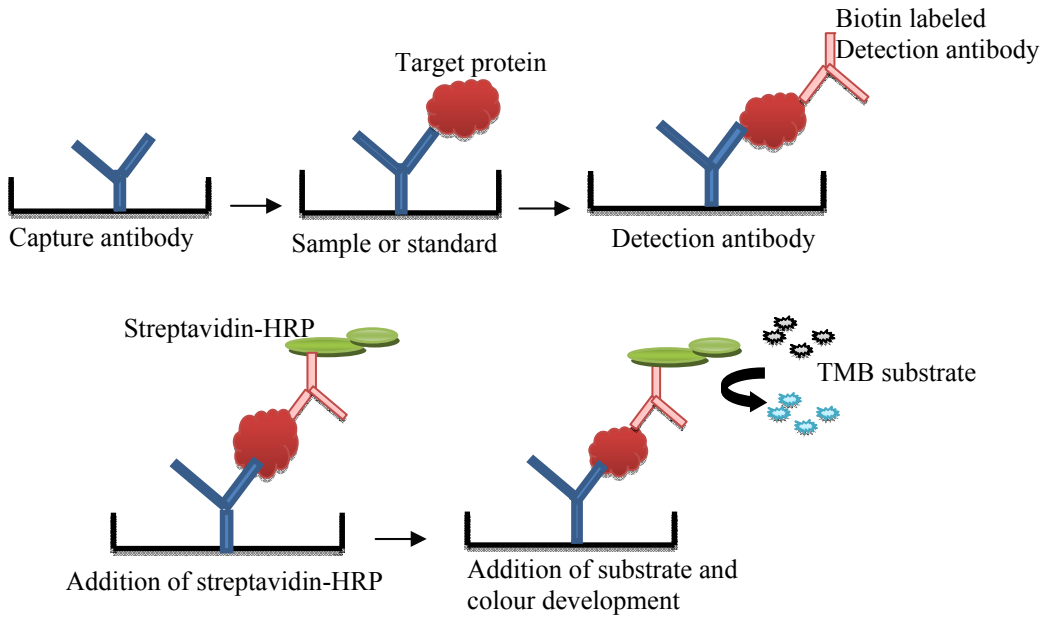


Figure 2.5.2 ELISA assay diagram, plate was coated with capture antibody and incubation for one hour. The non bound antibodies were washed away and then a samples or standard were added and incubated for 2 hours at room temperature, and then washed two times. The detection antibody was added followed by incubation for 2 hours. The non bound antibodies were washed away and substrate was added. The plate was read at 450 nm using spectrophotometer.

2.6 Isolation of $CD11c^+$ from BM DCs

Pure $CD11c^+$ DCs were isolated by a CD11c MicroBeads Column (Miltenyi Biotec). Non adherent BM cells were further purified by passing them through a CD11c MicroBeads column to isolate $CD11c^+$ cells. DCs were cultured as described in section 4.2.3 (About 20×10^6 BMDCs obtained from each mouse). 2×10^7 BM DCs were centrifuged at 200g for 10 minutes and the pellet was resuspended in 400µl of the buffer solution (PBS, pH 7.2, 0.5% BSA, and 2 mM EDTA), and 100µl of CD11c MicroBeads was then added and incubated for 15 minutes (2-8 ºC). The cells were washed using 1-2ml of the buffer solution and centrifuged at 200g for 10 minutes and resuspended in 500µl of the buffer solution and separated using a MACS separator. The column was washed 3 times with 500µl of the buffer solution and eluted cells

were collected in the waste tube as unlabeled cells (CD11c- cells). The column was removed from the separator and flushed with the buffer solution to obtain the labelled cells ($CD11c^+$) by firmly pushing the plunger into the column. $CD11c^+$ and $CD11c^-$ BM DCs were subsequently infected with live parasites and analysed for CD11c expression.

2.7 DCs expression of MHC class I, class II, CD11c, CD40, CD80, F4/80 and CD205

DCs were cultured as described in section 2.4.2 and were split into four groups. Two groups; control and test, were pulsed for 24 hours with 1μg/ml LPS to induce maturation. The other two groups; control and test, were similarly cultured but without LPS. The following day, BM-DCs were washed in serum free RPMI 1640 media, counted and divided into 2×10^5 cells/tube. The expression of MHC class I, class II, CD11c, CD40, CD80, F4/80 and CD205 DC cells was determined by staining with corresponding rat anti-mouse H_2-L^d, H_2 k^d, I/A-I/E, CD40 and CD80, F4/80, CD205 and rat anti hamster CD11c FITC labelled monoclonal antibodies (Table 2.7). Cells were fixed with paraformaldehyde and then analysed by flow cytometry.

Marker	**Primary antibody**	**control**
CD11c	Hamster anti mouse CD11c FITC	Hamster IgG FITC
CD205	Rat anti mouse CD205 FITC	Rat IgG2a FITC
CD40	Rat anti mouse CD80 FITC	Rat IgG2a FITC
CD80	Rat anti mouse CD80 FITC	Rat IgG2a FITC
F4/80	Rat anti mouse F4/80 FITC	Rat IgG2b FITC
MHC II (A/E)	Rat anti mouse I-A/I-E	Rat IgG2b FITC
MHC I (H_2-Kd,H_2-Ld)	Rat anti mouse H_2 Ld & H_2 kd	Rat anti mouse IgG2a FITC

Table 2.7 The Antibodies used to stain the bone marrow derived cells for phenotypinc characterisation.

2.8 Effect of autoclaved or live *L. mexicana* infection on the expression of MHC class I, MHC class II, CD11c, CD80 and CD40

DCs were prepared as previously described and split into two groups. The first group was infected with live *L. mexicana* and the second with autoclaved *L. mexicana* (the parasite was autoclaved at 121ºC under the pressure of 15 PSI for 20 minutes) 10 DCs for both live and autoclaved for 1 hour, 3 hours, 5 hours and 24 hours for MHC class I, II, (1 & 24 hours for CD11c, CD40 and CD80) as indicated for individual experiments. The expression of the MHC class I, II, CD11c, CD40 and CD80 molecules in the infected and autoclaved DC cells was determined by staining with rat anti-mouse H_2-L^d, H_2 k^d, I/A-I/E, CD11c, CD40 and CD80 all FITC labelled monoclonal antibodies, the cells were fixed with 1% of paraformaldehyde and then analysed by flow cytometry.

2.9 The effect of *Leishmania* infection and treatment with fungizone on the expression of surface molecules

DCs were prepared as previously described and then the DCs culture was split into three groups. The first group was infected with ten times the number of DCs with *L. mexicana* promastigotes for 24 hours as described above; the second group was similarly infected but treated after one hour with fungizone which killed the parasite infected DCs at a concentration of 7.5µg/ml then incubated for overnight at 37°C in 5% CO_2 atmosphere. The third group (non-infected) was used as control. On the following day DCs were washed, stained by antibodies for MHC class I, class II, CD11c, CD80 and CD40 molecules, the cells were fixed with 1% paraformaldehyde and analysed by flow cytometry.

2.10 Flow cytometry analysis of CD8+, CD4+ and CD3+ T cells of naïve splenocytes cultured with SLA

Splenocytes of Balb/c mice were flushed from the spleens using serum-free RPMI 1640 media. Cells were washed, counted and plated into 6 well tissue culture plates at a concentration of 1×10^6 per well. Three wells of splenocytes were then stimulated with 10µg/ml SLA2 (test group) and the other three wells were used as control (control group); splenocytes were incubated at 37°C in 5% CO_2 atmosphere. On day 5, splenocytes of each group were divided into four tubes at a concentration of 2×10^5/tube, and stained with FITC labelled anti CD3, CD4, CD8, and IgG2a (isotype control) as a negative control and analysed by flow cytometry (Table 2.10).

Markers	Primary antibody	control
CD3	Rat anti mouse CD3 FITC	Rat IgG2a FITC
CD4	Rat anti mouse CD4 FITC	Rat IgG2a FITC
CD8	Rat anti mouse CD8 FITC	Rat IgG2a FITC

Table 2.10 Antibodies of CD3, CD4 and CD8 T cell markers (Invitrogen).

2.11 Proliferation assay

Splenocytes from naïve or immunised Balb/c mice were flushed from the spleens by serum-free RPMI 1640 media. Cells were washed, counted and plated in 96 well tissue culture plates at a concentration of 5×10^4/200µl per well (in triplicate) in T cell media. Splenocytes were stimulated with (10µg/ml) whole SLA2 or SLA2 fractions (fr1, fr2, fr3, fr4, fr5 and fr6) and media used as a control. Cells were incubated at 37°C, 5% CO_2 atmosphere for 7 and 14 days. ^3H was added at 18 hours before termination to measure proliferation responses.

In some experiments DCs pulsed with antigens were used to stimulate splenocytes *in vitro*. DCs were generated as previously described in section 2.4.2. On day 6, DCs

were pulsed with 10µg/ml of whole SLA2 or SLA2 fractions and 4-6 hours later 1µg/ml LPS was added and incubated overnight. On day 7, DC cells were harvested, washed and pulsed again with 10µg/ml of corresponding SLA2 or SLA2 fractions (fr1, fr2, fr3, fr4, fr5 and fr6) for one hour and used for the stimulation of splenocytes (naïve and immunised). On the same day, spleens of naïve or immunised mice were harvested, Balb/c mice were immunised S.C. at the tail base with SLA2 or SLA2 fractions mixed with IFA at a concentration of 100µg/mouse of SLA2 or SLA2 fractions for 7 days. Cells were flushed from the naïve and immunised spleens by serum-free RPMI 1640, and then washed and resuspended in CTL media. Naïve and immunised splenocytes, were divided into four groups to be stimulated with each antigen (SLA2 or SLA2 fractions): (1) naïve splenocytes stimulated with SLA2 or SLA2 fractions (fr1, fr2, fr3, fr4, fr5 and fr6), (2) naïve splenocytes stimulated with DCs loaded with SLA2 or SLA2 fractions (fr1, fr2, fr3, fr4, fr5 and fr6) (3) naïve splenocytes stimulated with DCs alone as control and (4) media as additional control. The proliferation responses were assessed using the ^{3}H uptake assay. ^{3}H was added at 20µl per well and incubated 18 hours before harvesting. Cells were harvested on days 7 and 14 using 96 well proliferation filter plates and then dried for 1 hour, 40µl/well Microsint (Sigma) was added and the radioactivity was measured using a Top count scintillation counter.

2.12 Preparation of pCR T7/CT-TOPO *Ldcen-3*

The pCR T7/CT-TOPO- *Ldcen-3* vector (a kind gift from K. Nakhasi FDA, USA) was bulked up by transformation of *E. coli* as follows:

Day 1: DNA Transformation

In order to clone *centrin3* from pCR T7/CT-TOPO- *Ldcen-3* into pcDNA3.1/Hygro, both plasmids required bulking using *Escherichia coli* strain XLIB (prepared in lab). In a 1.5 ml Eppendorf, 1 µl of vector (pCR T7/CT-TOPO- *Ldcen-3* or pcDNA3.1/Hygro) was added to 200 µl of broth (see materials in appendix) containing competent XLIB bacteria, mixed gently and then incubated on ice for 30 minutes. Next, the bacteria were heat shocked for 3 minutes in a 42ºC water bath and then placed on ice for 5 minutes. Subsequently 500 µl of LB broth media were added to the bacteria/plasmid mix which was then incubated for 1 hour at 37ºC. After incubation, 200 µl of sample was transferred into 3 ml of LB media with 15 µl of ampicillin (50µg/ml) and this was incubated in an orbital shaker (Stuart) overnight at 37ºC.

Day 2: Plasmid Extraction

In duplicate, 1.5 ml of the cultured bacteria was transferred into a 2 ml Eppendorf tube and was centrifuged at 18,000 xg for 5 minutes. The supernatant was discarded and the pellet re-suspended in 100 µl of GTE (50mM glucose, 10mM EDTA, 25mM Tris.HCl, pH 8). This was mixed gently and then incubated on ice for 5 minutes. Then, 200 µl of solution (800 µl H_2O + 100 µl 10% SDS + 20 µl 10M NaOH) was added, the Eppendorf tube was mixed gently and the mixture was incubated on ice for 5 minutes. Subsequently 150 µl of potassium acetate KOAc (3 M KOAc, pH 4.8) to 300 ml ddH_2O, add KOAc 147.2 g, Acetic acid, cold 57.5 ml) volume adjusted to 500 ml with ddH_2O) was added to the mixture which was then mixed gently and incubated on ice for a further 5 minutes. The mixture was then centrifuged at 18,000 xg for 5 minutes, the supernatant was transferred into a clean Eppendorf tube and the pellet was discarded. Next, 800 µl of chloroform was added to the supernatant, mixed and then centrifuged at 18,000 xg for 5 minutes. The top layer of the mixture was

transferred into a clean Eppendorf tube and the bottom layer of chloroform was disposed of appropriately. Next, 1 ml of absolute ethanol was added and the mixture was incubated at room temperature for 15 minutes followed by centrifugation at 18,000 xg for 15 minutes. The supernatant was discarded and 500 µl of 70% ethanol was added, centrifuged for 5 minutes at 18,000 xg. The supernatant was discarded and the Eppendorf was inverted and allowed to dry for 30 minutes in a 37ºC incubator. When dry, the pellet was suspended in 20 µl of molecular grade water and 1 µl of RNAase was added to degrade unwanted RNA. The *Ldcen-3* gene construct was also sequenced by MWG-Biotech using the *Ldcen-3* primers (Table 2.13) and checked for mismatches against the Gene Bank sequence bank (http://www.JustBio.com). The presence of the gene insert in plasmid construct was usually determined by digestion with restriction enzymes.

2.13 Detection of *Ldcen-3* by PCR

PCR was performed by using a DNA Thermal cycler (Thermo Hybaid, USA). Primers for *Ldcen-3* were designed and obtained from MWG- Biotech (Table 2.13). 1µl of pCR T7/CT-TOPO was mixed with 5 µl of 10x PCR buffer, 0.8 µl, 10mM Deoxy nucleotide triphosphate (dNTP) and 3 µl (0.5µg) of *Ldcen-3* forward and reverse primers, 1.25 unit of thermostable Taq polymerase (Bioline), 1.5 mM $MgCl_2$ (Bioline), and water to a final volume of 50 µl. PCR was initiated by a melting step at 95ºC lasting for 5 minutes, followed by 33 cycles of denaturation at 95ºC for 1 minute, annealing at 58ºC for 1 minute and extension at 72˚ C for 45 sec. It was followed by a final extension step at 72ºC for 5 minutes. PCR products were visualized using a 1.5% (w/v) agarose gel containing 1 µg/ml of ethidium bromide (BDH Laboratories, UK), or 5µl Safe DNA run gel (Invitrogen).

Ldcen-3 primers	*Ldcen-3* Forward	5'-AGA GGC ATT CGT GTT CG-3`
	Ldcen-3 Reverse	5`AGG TTG ATC TCG CCA TCT TGA 3`

Table 2.13 Forward and reverse primers for *Ldcen-3* used for PCR and sequencing of *Ldcen-3*

2.14 Preparation of pCR T7/CT-TOPO empty vector

The pCR T7/CT-TOPO-*Ldcen-3* vector was digested by *Xba*I and *Hind* III restriction enzyme and separated by agarose gel electrophoresis. The heavier band corresponding to empty vector was then cut out of the gel and the DNA was extracted by DNA extraction kit (GeneFlow) according to the manufacturer's protocol. Two sides of the digested vector were ligated together by T4 DNA ligase (Promega). The ligation was set up by adding 0.5µl ligase, 1µl buffer and 6.5µl water to 2µl DNA (adjusted to 10µl). The ligated DNA was incubated at 4 ºC overnight.

2.15 Sub cloning of *Ldcen-3* in to pcDNA 3.1(-)

pCR T7/CT-TOPO-*Ldcen-3* and pc DNA3.1 (-) vectors (map is shown in Fig 6.2.1.1) were digested by *Hind* III and *Xba*I restriction enzymes and the digested products were separated by 1.5 % agarose gel. The *Ldcen-3* and pc DNA3 (-) bands were cut and extracted from the gel. The pc DNA3.1 (-) vector and the *Ldcen-3* DNA were ligated together. The direction of the gene in the vector was checked by cutting the new construct (pcDNA3.1 (-) *Ldcen-3* DNA) with the same restriction enzymes.

2.16 Transfection of CT26 tumour cells with *Ldcen-3*

Cell line	Description	Media	Source
CT-26	N-methylurethane-induced Balb/c murine colon carcinoma	DMEM+10% FCS	Prof Ian Hart (St Thomas Hospital)
CT-26 clone 25	Transfected with *LacZ*	DMEM+10% FCS	Prof Ian Hart (St Thomas Hospital)

Table 2.16 Cell Lines and their descriptions

2.16.1 Antibiotic sensitivity assay

CT26 tumour cells at a concentration of 1×10^6 cells/well were cultured in duplicate in 24 well plates in the presence of Geneticin (G418) (selective antibiotic required to be present in culture media to select transfected cells) from 50 to 900µg/ml. The cells were incubated at 37 ºC with 5% CO_2 for 10 days. The concentration of the antibiotic in which all the CT26 tumour cells died within 7-10 days was chosen for the selection of transfected cells (500µg/ml).

2.16.2 Transfection of CT26 with pcDNA3.1 (-) *Ldcen-3* DNA

CT26 tumour cells were transfected with the pcDNA3.1 (-)-*Ldcen-3* DNA construct by using lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction for adherent cells with slight modifications. CT26 tumour cells (Table 2.16) were cultured at 1×10^6/cells per well in 24-well plates, to produce 90% confluency on the day of transfection. Lipofectamine 2000 and the pcDNA3.1 (-)-*Ldcen-3* DNA construct were diluted in serum free DMEM media at 2µl/50µl and 0.8µg/50µl respectively and incubated at room temperature for 5 minutes. The diluted lipofectamine 2000 and DNA were mixed together and incubated again for 20-30 minutes at room temperature. The CT26 cell culture supernatant was gently removed

and the DNA-lipofectamine mixture was gently added followed by 4-6 hours incubation at 37 ºC in a 5% CO_2 atmosphere. 1ml/well DMEM media supplemented with 10% FCS was added. The media was replaced 16-24 hours later with fresh media containing 500µg/ml G418.

2.17 Subcloning of *LacZ* gene into pCR T7/CT-TOPO vector

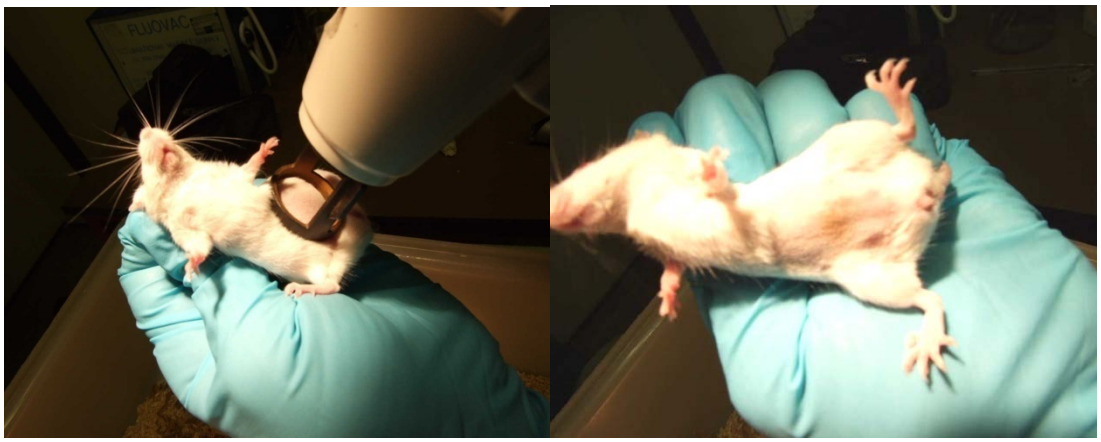
pCR T7/CT-TOPO-*Ldcen-3* and pcDNA 3.1 *myc LacZ* (-) were cut by *Xba*I and *Hind* III restriction enzymes, then the *lacZ* gene and the digested pCR T7/CT-TOPO vectors were ligated using a DNA ligase enzyme. The ligation was set up by adding 0.5µl ligation enzyme, 1µl buffer and 6.5µl water to 2µl DNA. The ligated DNA was incubated at 4 ºC overnight.

2.17.1 Expression of β-gal using pCR T7/CT-TOPO –*LacZ* construct

The β-Galactosidase staining kit (Sigma) was used to determine the expression of *LacZ* following transient or stable transfection of plasmids encoding *LacZ*. CT26 tumour cells were plated at a concentration of 1×10^6/cells per well, in serum free DMEM media. CT26 cells were transfected with the pCR T7/CT-TOPO-lacZ DNA construct as detailed in section 2.14.2. On day three following the transfection cells were washed twice with PBS then fixed with 1ml glutaraldehyde (0.05%) for 15 minutes at 37ºC. Cells were again washed twice with PBS and 1ml of X-Gal solution/well was added and incubated for overnight. Transfected CT26 are tested under the microscope for the development of blue stain which indicating the expression of β-galactosidase in transfected cells.

2.18 Coating of gold particles by DNA

The DNA construct was coated onto 1.0 Micron gold particles (Biorad, Hemel Hempstead, Hertfordshire, UK) using manufacturer's instruction and administered by Helios gene gun (Biorad). About 200 µl of spermidine was added to 16.6 µg of gold followed by sonication. A total of 36µg of DNA was added followed by the addition of 200 µl of 1M calcium chloride and incubated at room temperature for 10 minutes. Tubes were centrifuged at 16,000 xg for 1 minute and gold particles were resuspended in ethanol (Sigma). After repeating the above step 2 more times, particles were resuspended in 0.025mg/ml of poly-vinyl-pyrollidone (PVP) in ethanol. During these steps, the plastic tube was dried for 15-20 minutes using nitrogen gas. The resuspended gold particles were loaded into the dried tube using a syringe which was then placed on the roller/dryer (Biorad) followed by incubation for 15 minutes. Ethanol PVP was gently removed using the syringe and the tube was rotated on the roller along with nitrogen gas being passed through it for 5 minutes. Bullets were then cut using a guillotine and stored at 4ºC until used for immunisation (Fig 2.18).



1 **2**

Figure 2.18 Mouse being injected with gene gun; Particle mediated epidermal delivery of a *Leishmania* DNA vaccine, 1 injecting Balb/c mouse with DNA by gene gun, 2: Balb/c injected with gene gun.

2.19 RNA Extraction

The CT 26 cells of transfected and non transfected were centrifuged at 1500 xg for 4 minutes at 4ºC. The supernatant was discarded and the pellet was resuspended in 1ml RNA STAT-60. The cells were homogenized and incubated for 5 minutes at room temperature. 0.2 ml of chloroform was added to the cell suspension and mixed vigorously. The mixture was then transferred to two 1.5 ml Eppendorf tubes and incubated at room temperature for 2 minutes before centrifugation at 18,000 xg for 15 minutes at 4ºC. The upper phase containing the RNA was collected very carefully with a micropipette and transferred into another Eppendorf tube. 0.5 ml isopropanol was added to precipitate the RNA which was incubated at room temperature for 10 minutes. The vials were centrifuged at 14,000 xg for 10 minutes at 4ºC. The supernatant was discarded and the RNA pellet was washed with 70% ethanol. The samples were centrifuged at 7,500 xg at 4ºC for 5 minutes. The supernatant was discarded and the RNA pellet was air dried for 10 minutes. The pellet of both the vials were then resuspended in 30 µl distilled water and stored at -80ºC.

2.20 RT PCR

The concentration of extracted RNA was measured using a UV spectrophotometer at 260/280 nm wavelength ratio for both the transfected and non transfected cells. To initiate the reaction 1 µl of oligo (dT_{15}) primer to 9 µl of RNA for each of the transfected and non transfected cells was added in two separate Eppendorfs. The vials were incubated at 70ºC for 5 minutes and cooled on ice for 2 minutes. The mixture was prepared by adding the following reagents.

Moloney Murine Leukemia Virus (M-MLV) 5 X buffer	5 µl
Deoxynucleotide (dNTP) mix (5mM)	1 µl
R Nasin (Ribonuclease inhibitor; 40U/ µl)	0.7µl
M-MLV Reverse Transcriptase (M-MLV RT; 200U/ml)	1 µl
Distilled water	7.3µl

15 µl of the above mixture was mixed with 10 µl of RNA and incubated at 39.2ºC for 80 minutes.

The PCR sample was prepared in four 1ml Eppendorfs. The following were added to the Eppendorfs: 15 µl of 10x HF (High-Fidelity) PCR buffer, 4.5 µl of 50 mM $MgCl_2$, 2.4 µl dNTP, 0.75 µl of Biotaq DNA polymerase (Bioline, Germany) and 115.5µl of distilled water (Nanopore Diamond water purifier Barnstead). 3 µl of forward primer (seq1) and 3 µl of reverse primer (seq 2) (Table 2.20) were added to two of the tubes. The other two tubes contained 3 µl each of forward and reverse murine GAPDH (A mouse house keeping gene used as positive control) (primers). 49µl of the sample from each of the Eppendorf tubes containing the *Ldcen-3* primers were transferred to 5 PCR tubes and the sample containing GAPDH forward primers and reverse primers were transferred to 4 PCR tubes. The 2 sets of cDNA from transfected and non transfected cells (1µl) were added to the PCR tubes. 1 µl of the cloned product (*Ldcen-3*) was added to one of the PCR tubes, which served as the positive control. Two PCR tubes were devoid of DNA which served as the PCR controls for *Ldcen-3* and GAPDH respectively. The samples and controls were subjected to the following conditions in the PCR thermocycler. PCR was initiated by a melting step at 95˚ C lasting for 5 minutes, followed by 33 cycles of denaturation at 95˚ C for 1 minute, annealing at 58˚ C for 1 minute and extension at 72˚ C for 45 sec. It was followed by a final extension step at 72˚ C for 5 minutes.

Application	Name	Sequence
GAPDH	mGAPDH Forward	5'-ACTCCACTCACGGCAAATTC-3'
	mGAPDH Reverse	5'-CCTTCCACAATGCCAAAGTT-3'
Ldcen-3	Forward primers	5`AGA GGC ATT CGT GTT CG-3`
	Reverse primers	5`AGG TTG ATC TCG CCA TCT TGA-3`

Table 2.20 Primers used for PCR, sequencing of mouse GAPDH and *Ldcen-3*

2.21 Immunisation protocols

2.21.1 Immunisation with pcDNA3.1 (-)-*Ldcen-3* and pCR T7/CT TOPO *Ldcen-3* constructs by gene gun.

All animal injections mentioned in this thesis were conducted by Dr Selman Ali. Six Balb/c mice per group were immunised twice with 1µg DNA of each vector coated on gold particles by gene gun on days 0 and 14 on a shaved area of the abdomen (Fig 2.18). Seven days after the last immunisation mice were challenged with 2×10^6 live *L. mexicana* promastigotes I.D. on the back at about 1 cm from the tail base. A control group of 6 mice was administered with gold particles coated with 1µg of empty plasmid by gene gun. An additional control group of 4 mice was immunised by PBS. Mice were monitored at least twice a week following the challenge with the parasite.

2.21.2 Immunisation of mice with SLA1 and SLA2

Two groups of 6 Balb/c mice were immunised S.C. at the tail base with 100µg SLA mixed with IFA (total volume of 200µl per mouse) or 200µl PBS twice (for control group) at 2 week intervals. Two weeks later, all mice were challenged with 2×10^6 *L. mexicana* promastigotes in 50µl PBS. The mice were monitored regularly twice a week.

2.21.3 Immunisation with SLA, DC and DCs pulsed with SLA

Three groups of 6 female Balb/c mice were either immunised with 1×10^6 SLA-pulsed or control DCs or, 100µg/mouse SLA alone. A fourth group of 4 mice was injected with PBS and used as additional control. All DC immunisations were administered I.D. on the right flank twice at two week intervals. Two weeks later all mice were challenged with 2×10^6 *L. mexicana* promastigotes and monitored regularly twice a week.

Statistica analysis: T Test was used to measure P value in all experiments presented in this thesis comparing between control to test samples (T test P*≤0.05, P**≤0.01, P***≤0.001) and P*≤0.05 considered significant. Standard deviation was also determined for all experiments in this study. All fowcytometry data presented in this study were based on analysis using WinMDI, a software available free on the web (http://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm).

Chapter 3 Result

Immune responses to *Leishmania* Antigens

3.1 Introduction

In order to develop an effective vaccine against Leishmaniasis, it is important to understand the mechanisms of the immune response to *Leishmania* infection, so that vaccines can be engineered to induce a protective response rather than one that exacerbates the infection (Kedzierski, 2010). The immune response to *Leishmania* infection is dependent on the species of the parasite and the genetic background of the host; therefore some mouse strains are resistant (C57BL/6) whilst others are susceptible (Balb/c). Resistance is conferred by Th 1 cells whereas susceptibility is conferred by Th 2 cells (Awasthi *et al*., 2004; Scott & Hunter, 2002).

There are a number of key factors that control the immune response to *Leishmania,* the outcome of infection being largely dependant on the ability of the host to mount a protective Th1 response versus the ability of the parasite to evade and manipulate the host's immune system (Vanloubbeeck and Jones 2004; Richard *et al*., 2010). It has been suggested that the immune response to *Leishmania* (Th1 or Th2) is dependent on the type of *Leishmania* antigen presented and recognised by T cells therefore, Th1 responses may be initiated by antigens different to those inducing Th2 responses (Awasthi *et al*., 2004). However, animal studies have indicated that the same parasite epitope may induce a Th1 or Th2 response, reflecting susceptibility to the disease (Piscopo *et al*., 2007). A recombinant *Leishmania* vaccine, using a combination of antigens expressed by several *Leishmania* species offers protection against mixed infections (Bastrenta *et al*., 2003; Berberich *et al*., 2003). Campos-Neto *et al*., (2001) have shown that the recombinant *Leishmania* antigens TSA and LmSTI1 induced high protection in both murine and rhesus monkey models of human cutaneous

Leishmaniasis. Macrophages and effector cells, DC, T-helper cells (CD4+ T cells), CD8+ T cells, NK cells and cytokines, are all known to play important roles in the regulation of immune response to *Leishmania* infection (Liese *et al*., 2008).

The first attempt to characterise *Leishmania* antigens used a biochemical approach to purify parasite proteins or membrane fractions or secreted proteins mostly from promastigotes (Devault & Banuls, 2008). The surface proteins of the parasite appear to be more important in the initiation of *Leishmania* infection and are potential targets in developing a vaccine strategy that can be used for serodiagnosis of canine and human VL. The mixture of naturally excreted antigens, purified from the supernatant of *L. infantum* promastigotes "LiESAp" was shown to provide immunity in dogs infected with *L. infantum* (Lemesre *et al*., 2007). Also, two protein fractions of different molecular weight, Ric-2 and Ric-1, secreted by *L. infantum* promastigotes (Ric-1 contains high molecular weight excreted proteins and Ric-2 the low molecular weight ones) were shown to stimulate different immune responses, mostly by the modulation of the Th1/Th2 cytokine balance (Rosa *et al*., 2005). Similarly, an anti-gp63 antigen specific Th1 response was induced in mice immunised with bone marrow derived dendritic cells (BMDCs) pulsed with a gp63 peptide (Tsagozis *et al*., 2004). The gp46 was also evaluated in different models: in *L. major*, the resistance depends on the source of the gp46 antigen protein and the immune responses induced by the adjuvant (Handman *et al*., 1995), however, susceptible Balb/c mice immunised with an attenuated recombinant virus vaccine expressing *L. amazonensis* gp46 induced protection against *L. amazonensis* infection (McMahon-Pratt *et al*., 1993; Launois *et al*., 2008). The *L. donovani* Kinetoplastid Membrane Protein 11 (KMP-11) is a surface membrane glycoprotein associated with the LPG and is a strong T cell stimulating factor (Jardim *et al*., 1995).

Guedes *et al*., (2010) have reported that intramuscular inoculation of Balb/c mice with *L. amazonensis* promastigote antigens (LaAg) enhanced the susceptibility to CL. Mice were immunised intramuscularly with LaAg that was pre-treated with serine or cysteine protease inhibitors (SPi and CPi) before challenge with *L. amazonensis*. The resistance was linked to reduced production of IL-10 and TGF-β of the lesion-draining lymph node cells in response to parasite antigens in comparison to the control. *In vitro*, soluble proteases from LaAg triggered IL-10, IL-4 and TGF-β production by immune cells. This clearly suggests that, the outcome of immunistion is influenced by the type of triggered cytokines.

Potent vaccines against *Leishmania* parasite should ideally contain different antigens that stimulate both CD4+, CD8+ and IFN-γ responses biased towards Th1 rather than Th2 (Ramirez *et al*., 2010). SLA was shown to protect Balb/c mice against challenge with *Leishmania* infection, and the produced immunity was associated with the induction of IFN-γ, which caused macrophage activation (Bottrel *et al*., 2001). Another study by Sharma *et al*., (2006) has shown that *Leishmania donovani* promastigote soluble antigens (SLAg) encapsulated in non-phosphatidylcholine (non-PC) liposomes (escheriosomes) prepared from *E. coli* lipid induced strong humoral and cell mediated immune responses both in hamsters and Balb/c mice. Immunisation of Balb/c mice with SLA or SLA administered with Incomplete Freund's adjuvant (IFA-SLA) enhanced $CD8^+$ cytotoxic T lymphocyte responses. SLA also induced the release of mixed Th1 and Th2 cytokines in the immunised Balb/c mice. Therefore, in this study the immunogenicity of SLA and SLA fractions from *L. mexicana* was investigated in order to establish the immunogenicity of each fraction in the Balb/c mouse model, the summarizing of experimental work in this chapter shown in (Fig 3.1).

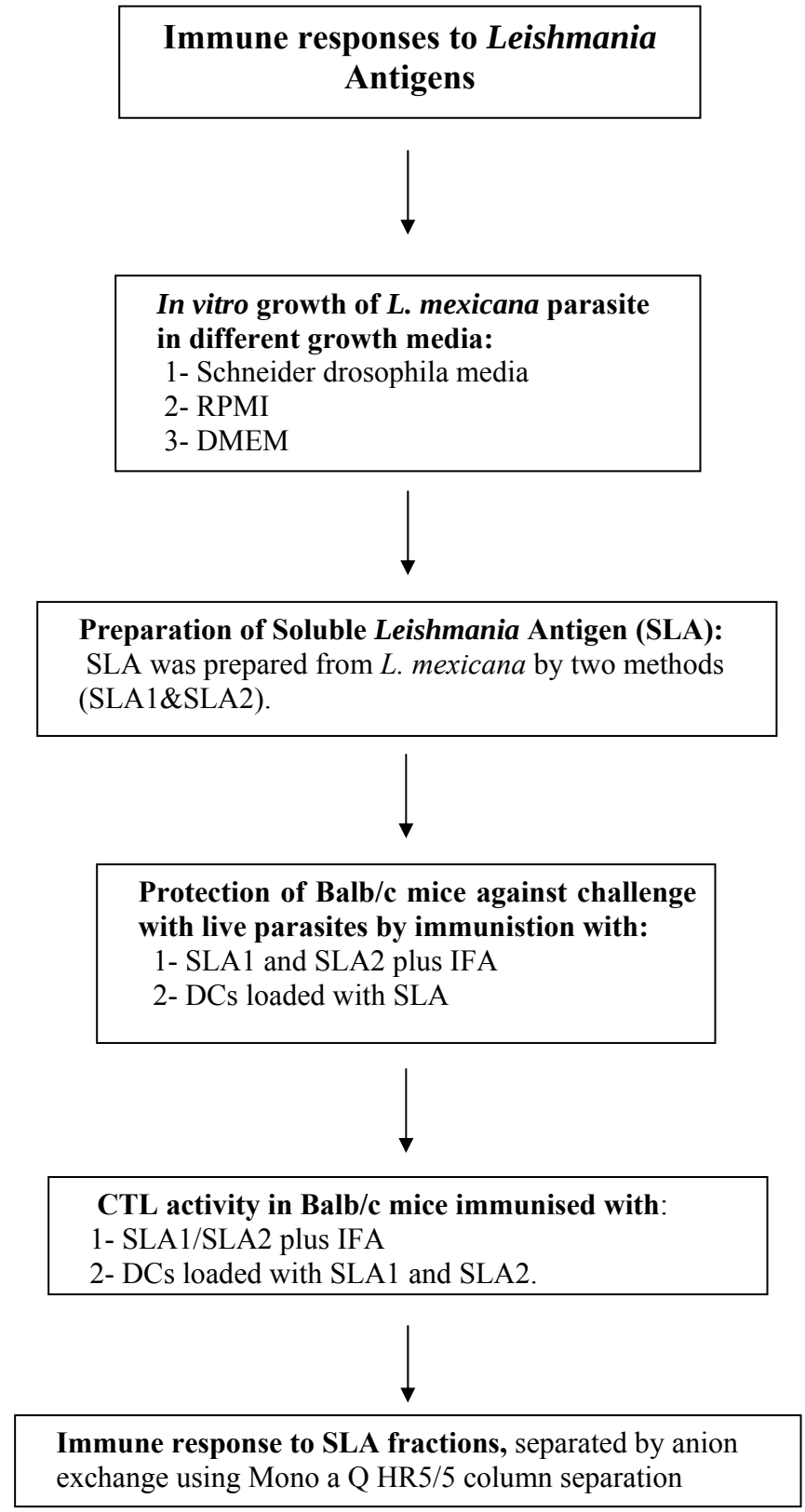


Figure 3.1 A flow chart summarizing the experimental work in this chapter.

3.2 Results

3.2.1 Growth of *L. mexicana* parasite in different growth media

In this study *L. mexicana* growth characteristics were investigated *in vitro* in three different growth media: Schneider drosophila media, RPMI and DMEM over a period of seven days. The growth rate of the *L. mexicana* parasite was different depending on the type of growth media used. The highest number of parasites/ml was achieved using Schneider medium. The log phase for growth in RPMI was up to 6 days which was longer than that of Schneider medium (up to 4 days). The growth of *Leishmania* in RPMI medium is therefore slower as a low number of parasites/ml was obtained over a longer period of time. DMEM medium did not support *Leishmania* growth; the number of parasites remained the same for up to 3 days after which the parasites started to decrease in number (Fig 3.2.1).

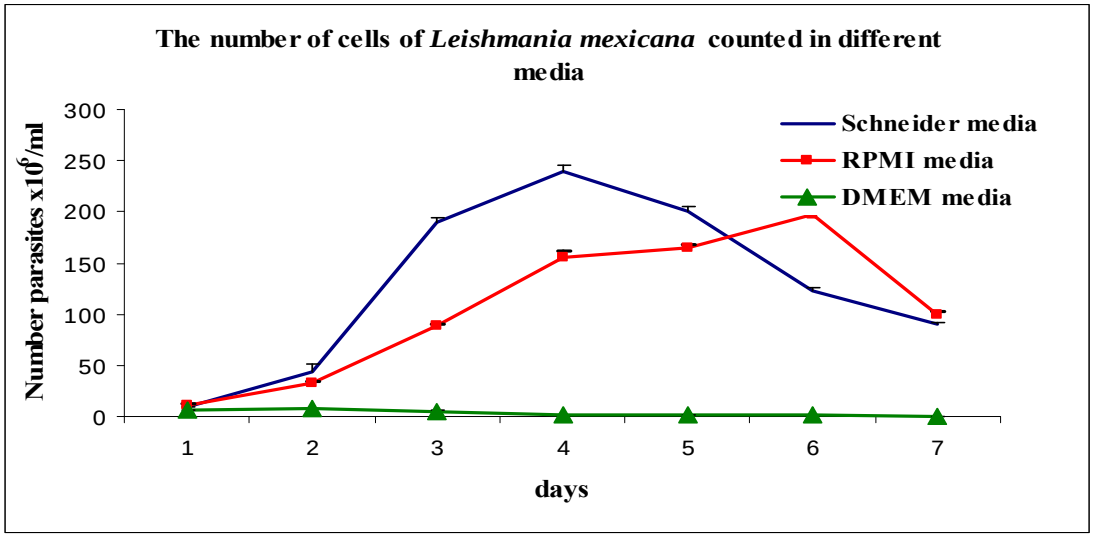


Figure 3.2.1 Growth of *L. mexicana* parasite in different growth media
A culture of 1×10^6/10ml of *L. mexicana* parasites in T25TC flask was initiated in 3 different culture media: Schneider drosophila media, RPMI and DMEM. 10µl of the parasites culture was diluted twice in 90µl of 1% paraformaldehyde and counted daily for up to 7 days using a haemocytometer. The graph represents 3 independent experiments.

3.2.2 Preparation of Soluble *Leishmania* Antigen (SLA)

In this study Soluble *Leishmania* Antigens (SLA) were prepared by two different methods, and immune responses to these antigens; SLA1 and SLA2 were investigated using a Balb/c mouse model. The Schneider medium was used to culture *Leishmania* parasites (see chapter 2 methods).

3.2.3 Detection of *L. mexicana* gp63 in SLA1 and SLA2

Gp63 protein or Leishmanolysin is the most abundant protein on the surface of the promastigote form of the *Leishmania* parasite. Gp63 assists the parasite to infect macrophages and also has shown potential as a protective immunogenicity in mice (Yao, 2010). The presence of gp63 protein in SLA1 and SLA2 preparations was determined by western-blotting using rabbit anti *L. mexicana* gp63 antibodies. The result demonstrates the presence of gp63 bands in SLA2 compared with SLA1 (Fig 3.2.3).

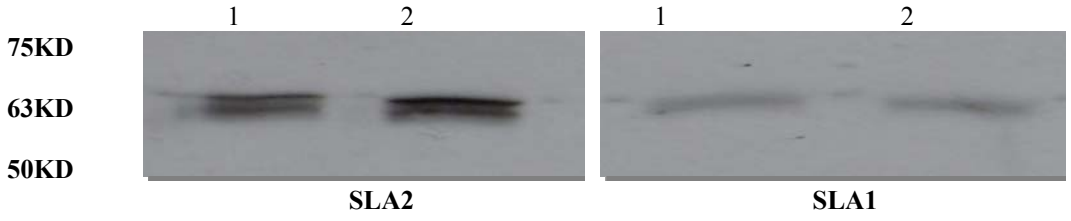


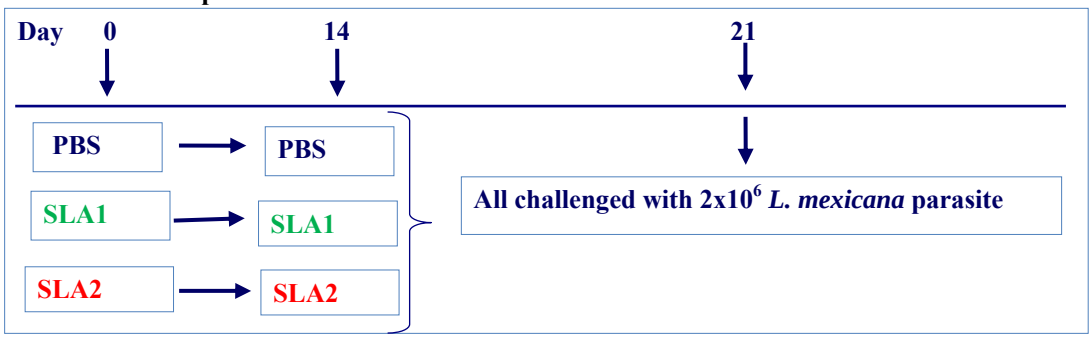
Figure 3.2.3 Detection of *L. mexicana* gp63 in SLA1 and SLA2
L. mexicana parasites were used to prepare SLA1 and SLA2 (chapter 2 methods). The SLA preparations were analysed for the presence of *L. mexicana* gp63 by western blotting using rabbit anti - *L. mexicana* gp63 protein. Lanes 1 and 2 represent two preparations produced at different times.

3.2.4 Protection induced by immunisation with SLA1 and SLA2

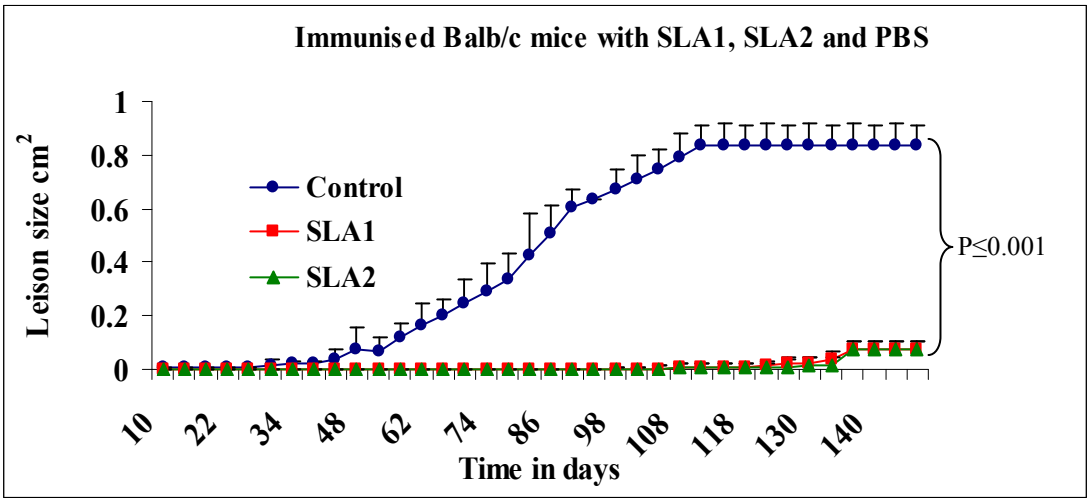
The immunogenicity of SLA1 and SLA2 was tested in a protection experiment in Balb/c mice challenged with *L. mexicana.* Six Balb/c mice were immunised S.C. with

100µg/mouse of SLA1 or SLA2 mixed with IFA, twice at two week interval. Seven days after the second immunisation all mice were inoculated with 2×10^6 *L. mexicana* promastigotes on the back, approximately 1cm from the tail base. The results clearly demonstrate that mice immunised with SLA1 or SLA2 were significantly protected against challenge with live *L. mexicana* promastigotes. Where 4 out of 6 mice, whether immunised with SLA1 or SLA2 remained free of lesions throughout the experiment (Fig 3.2.4-A&B). All animals in the control group (4 mice) developed progressive cutaneous lesions at the site of infection (Fig 3.2.4-C).

A: Immunisation protocol



B:



C:

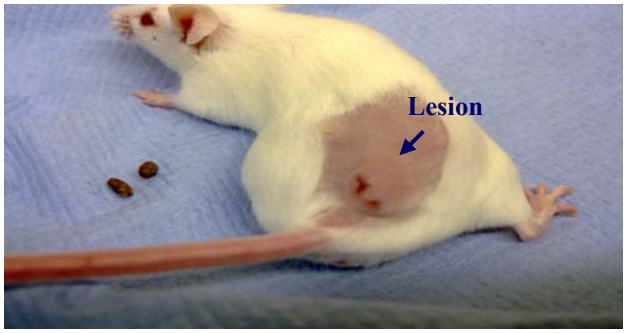


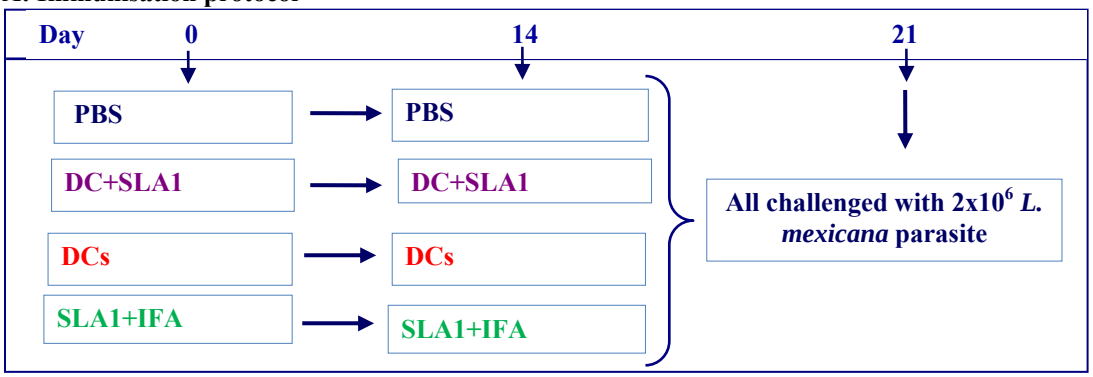
Figure 3.2.4 Protection against challenge with *L. mexicana* induced by immunisation with SLA1 or SLA2

A: Immunisation protocol; B: Protection induced by immunisation with SLA1 and SLA2: Two groups of six Balb/c mice were immunised S.C. with 100µg/mouse of SLA1 or SLA2 mixed with IFA and four control mice were injected with PBS. Seven days after the second immunisation, all mice were inoculated with 2×10^6 *L. mexicana* promastigotes. Mice were monitored twice a week; the graph represents 2 independent experiments. Bars represent the standard deviation of the mean, n=6. Protection induced by immunisation with SLA1&2 were statistically significant when compared with control using T test, p***≤0.001.**C: *L. mexicana* lesion after infected with 2×10^6 promastigotes in control mice:** All mice in the control group developed progressive cutaneous lesions at the site of infections.

3.2.5 Protection induced by immunisation with SLA1 and SLA1 loaded DCs

Groups of six female Balb/c mice were either immunised S.C. with 100µg SLA1/mouse mixed with IFA or 1×10^6 DCs alone or DCs loaded with 100µg/mouse SLA1 or PBS (see chapter 2 methods). The results clearly show that mice immunised with SLA1 mixed with IFA but not with DCs loaded with SLA1 were significantly protected against challenge with live *L. mexicana* parasite where 50 % (3 out of 6 mice) of mice remained lesion free, compared with DCs loaded with SLA1 and control groups (4 mice) where all the mice developed lesions (Fig 3.2.5). Surprisingly, mice immunised with DC alone had less lesion progression in comparison to mice given DCs loaded with SLA1. I.D route was chosen for DCs immunisation as they are naturally present in the dermis.

A: Immunisation protocol



B:

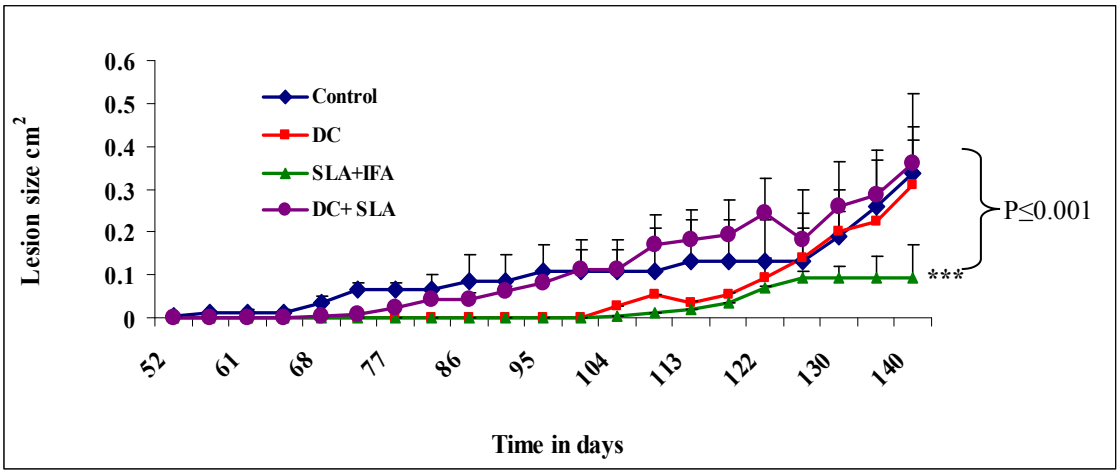


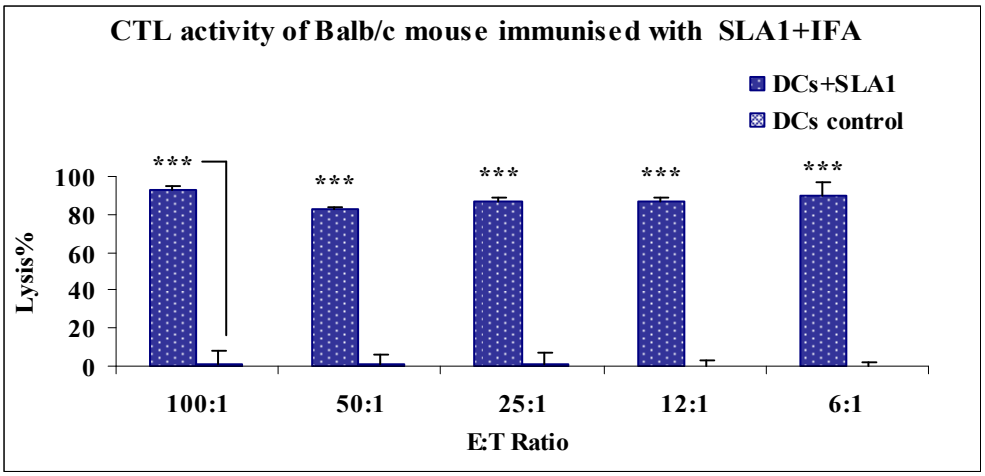
Figure 3.2.5 Protection induced by immunisation with SLA1 plus IFA or SLA1 loaded DCs
A: Immunisation protocol; B: Protection induced by immunisation with SLA1 or SLA1 loaded DCs: Four groups of six Balb/c mice were immunised S.C. with 100µg/mouse of SLA1 mixed with IFA or I.D. with 1×10^6 DCs alone or loaded with SLA1 or with PBS. Seven days later all mice were inoculated with 2×10^6 *L. mexicana* promastigotes. Mice were monitored twice weekly. The graph represents 2 independent experiments. Bars represent the standard deviation of the test mean n=6 control mean n=4. Protection induced by immunisation with SLA+IFA were statistically significant when compared with control using T test, p***≤0.001.

3.2.6 CTL activity in Balb/c mice immunised with SLA1/SLA2 plus IFA

Balb/c mice were immunised S.C. at the base of the tail with either 100µg/mouse SLA1 or SLA2 with IFA. Splenocytes were harvested and cultured *in vitro* for 5 days together with blast cells pulsed with LPS and corresponding SLA (see chapter 2 methods). On day 5, the splenocytes were used as effector cells in a standard 4-hour cytotoxicity assay against non-adherent DCs loaded with the corresponding SLA antigen preparations. Splenocytes from Balb/c mice immunised with *L. mexicana*

SLAs (SLA1 or SLA2) in combination with Freund's adjuvant (IFA) and mice immunised by DC loaded with SLAs induced potent $CD8^+$ cytotoxic T lymphocyte (CTL) response compared to control group, against DC targets loaded with corresponding SLA. However maximum cytotoxity was even observed at the minimum effector to target ratio of 6:1 for SLA1 but at 25:1 for SLA2 (Fig 3.2.6-A&B compared with Fig 3.2.6-C&D). This is not due to the non specificity of the assay since including anti CD8 antibodies significantly inhibited the cytotoxic responses in this assay (data not shown).

(A):



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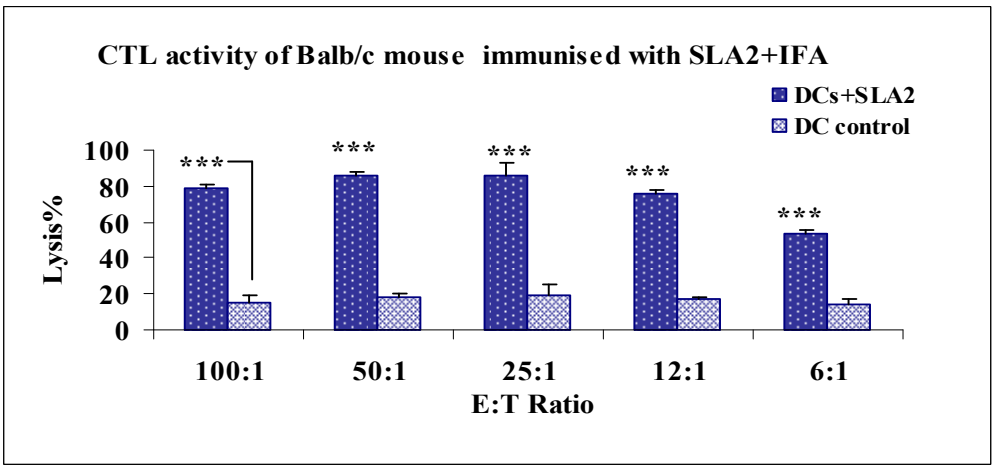
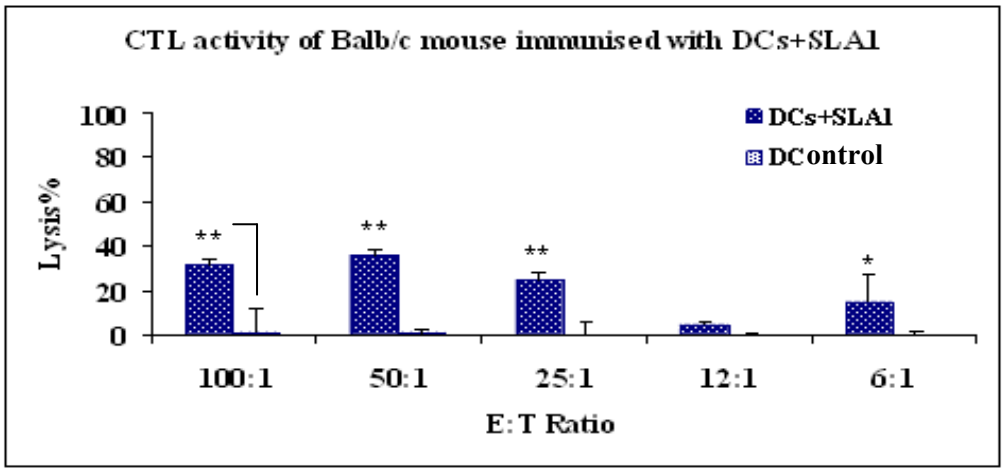


Figure 3.2.6 CTL activity of Balb/c mice immunised with SLA1 or SLA2
A: Balb/c mice immunised with SLA1; B Balb/c mice immunised with SLA2: Balb/c mice were immunised s.c with 100µg/mouse SLA1 or SLA2 plus IFA. Splenocytes were cultured *in vitro* for 5 days together with blast cells pulsed with LPS and corresponding SLA. On day 5 splenocytes were used as effector cells in a standard 4-hour cytotoxicity assay against DCs pulsed with corresponding SLA. The graph represents 3 independent experiments n=9, the response induced by immunisation with SLA+IFA were statistically significant when compared with control using T test P***≤0.001.

Results in Fig 3.2.6-C&D clearly revealed that immunisation of mice with DCs loaded with *L. mexicana* SLA1 or SLA2 also induces specific CTL activity against DCs loaded with corresponding SLA. However, the mice immunised with *L. mexicana* SLA1 or SLA2 plus IFA induced much higher CTL activity compared with mice immunised with DCs loaded with SLA1 and SLA2.

(C):



(D):

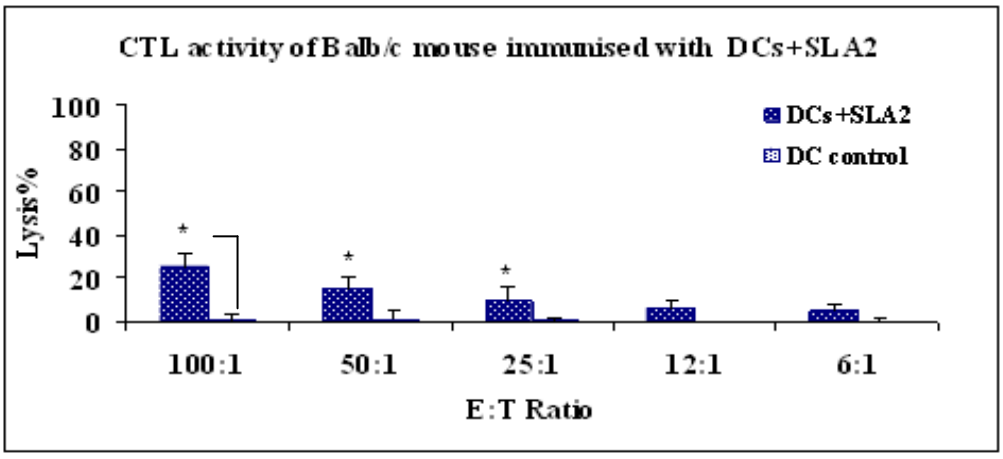


Figure 3.2.6 CTL activity of Balb/c mice immunised with DCs pulsed with SLA1 or SLA2
C: Balb/c mice immunised with DCs pulsed with SLA1, **D: Balb/c mice immunised with DCs pulsed with SLA2:** Balb/c mice were immunised I.D. with 2×10^6 DCs loaded with SLA1 or SLA2 per mouse. After two weeks the mice were sacrificed and their splenocytes were cultured in vitro for 5 days together with blast cells pulsed with LPS and corresponding SLA. On day 5 they were used as effector cells in a standard 4-hour cytotoxicity assay against DCs pulsed with corresponding SLA. The graph represents 3 independent experiments n=9, the response induced by immunisation with DCs+SLA1and DCs+SLA2 were statistically significant when compared with control using T test P*≤0.05, p**≤0.01.

3.2.7 Fractionation of Soluble *Leishmania* Antigen (SLA)

Both SLA1 and SLA2, used with IFA for immunisation, produced similar *in vivo* protection and CTL activity in immunised mice. In this study SLA2 was further fractionated and analysed. Six sub fractions of promastigote derived SLA were separated by anion exchange using fast performance liquid chromatography (FPLC) Mono Q HR5/5 column separation. The elution profile of the separated fraction using a linear NaCl gradient from 0 to 1M is shown in (Fig 3.2.7). Six major peaks were observed from promastigote SLA which were subsequently eluted by NaCl gradient.

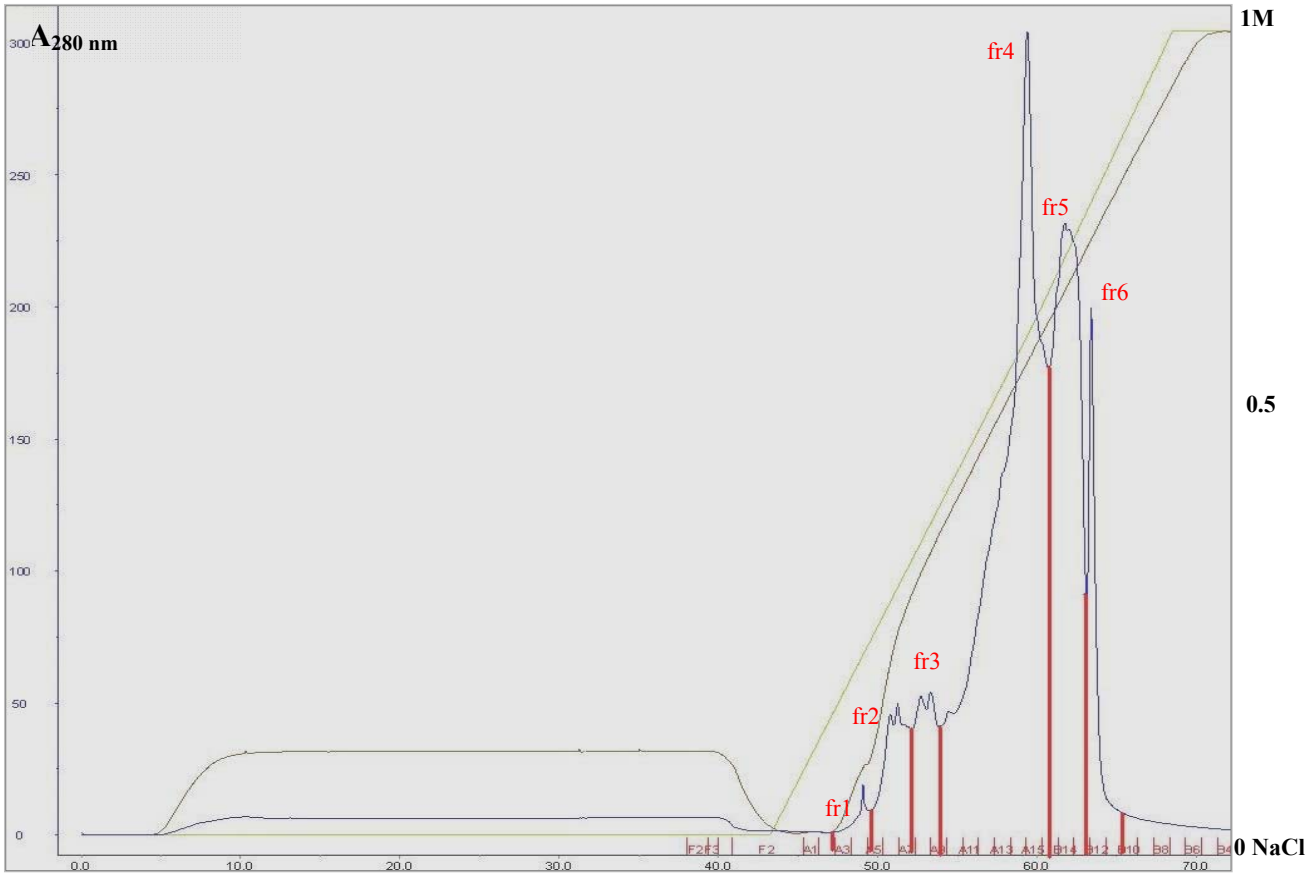


Figure 3.2.7 Isolation of promastigote SLA by fast performance liquid chromatography (FPLC) anion-exchange chromatography
Samples of 10 ml SLA promastigote SLA in buffer A (100 mM Tris, 1 mM EDTA pH 8.0) were loaded into a Mono Q HR5/5 column. The SLA was eluted using a NaCl gradient of 0-100% in buffer B (buffer A with 1 M NaCI) at a flow rate of 0.5ml/min; proteins were detected using UV at 280 nm Red: peaks of fractions, green: buffer B and black: gradient NaCl.

3.2.8 Detection of *L. mexicana* gp63 in fractions of Soluble *Leishmania* Antigen (SLA)

All fractions of promastigote SLA were analysed by western-blotting for the presence of the gp63 protein using rabbit anti gp63 monoclonal antibodies. According to the western-blotting analysis, a strong 63-kD band was only detected in whole SLA2 compared with a weak band in fractions 2 and 3, and no band in fractions 1, 4, 5, and 6 (Fig 3.2.8). The gp63 bands were weak or not present in all the fractions after separation of SLA2, possibly due to dilution of gp63 between the fractions. The protein content of each fraction was measured prior to loading on the gel and an equal concentration was loaded into each well.

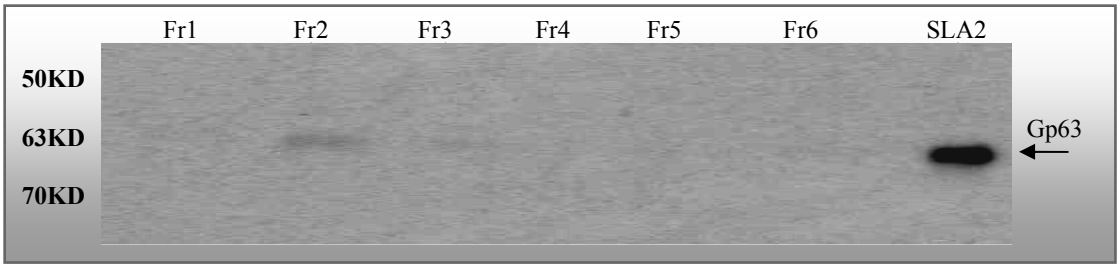


Figure 3.2.8 Detection of *L. mexicana* gp63 in SLA fractions
L. mexicana parasites were used to prepare SLA fractions (chapter 2 methods). The SLA fraction preparations were analysed for the presence of *L. mexicana* gp63 by western blotting using rabbit anti *L. mexicana* gp63 protein by persulphate) was added on top of the resolving gel and the comb was inserted in it. To prepare the samples, 33µl of 1x reducing sample buffer was added to 100µg according to the concentration of each sample (SLA2 and SLA2 fractions) and then 20 µl from each sample were loaded in the gel.

3.2.9 CTL activity of Balb/c mice immunised with SLA2 and SLA2 fractions

This study demonstrated that immunisation of mice, with soluble *Leishmania* antigens extracted from the *L. mexicana* promastigote, protected them against challenge with live *Leishmania mexicana*. In an attempt to distinguish the immunogenic antigens within the SLA2 preparation, SLA2 was separated into distinct fractions by anion-exchange chromatography, and the ability of each fraction

to stimulate immunity was tested by immunisation of Balb/c mice. Balb/c mice were immunised S.C. at the tail base either with SLA2, or SLA2 fractions with IFA at a concentration of 100μg/mouse. Splenocytes were harvested one week after immunisation and cultured *in vitro* for 5 days together with blast cells pulsed with LPS and corresponding SLA or SLA fraction. On day 5, the splenocytes were used as effectors in a standard 4-hour cytotoxicity assay against non-adherent DCs loaded with corresponding SLA2 fraction. Splenocytes from Balb/c mice immunised with *L. mexicana* SLA fractions in combination with IFA induced a potent $CD8^+$ cytotoxic T lymphocyte (CTL) response as compared to the control, but SLA induced the highest CTL compared with all other fractions (Fig 3.2.9), although all of the individual fractions showed the ability to promote significant CTL killing.

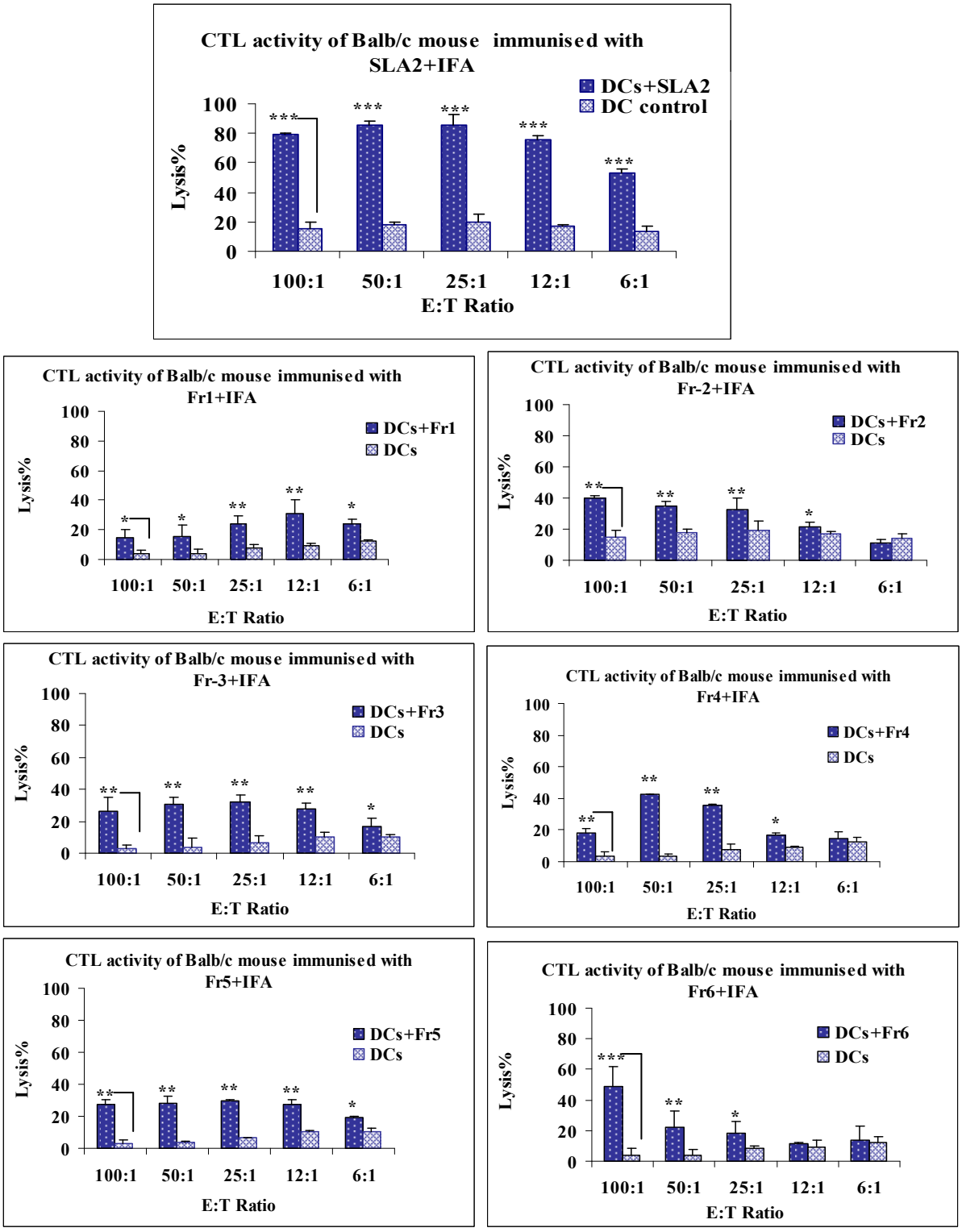


Figure 3.2.9 CTL activity of Balb/c mice immunised with SLA fr1-6
Balb/c mice were immunised S.C. with 100µg/mouse of SLA2 and SLA2 fractions 1 to 6. Non-adherent DC pulsed with corresponding SLA or SLA2 fractions 1-6 were used as target cells. Splenocytes were cultured *in vitro* for 5 days together with blast cells pulsed with LPS. On day 5 they were used as effector cells in a standard 4-hour cytotoxicity assay. Each graph represents 6 mice in 3 independent experiments, 2 mice in each experiment. Results were statistically significant where DCs+SLA fraction was compared with control DCs using T test P*≤0.05, p**≤0.01, p***≤0.001.

3.3 Discussion

Immunity in Leishmaniasis is mediated by the stimulation of Th1 cells to produce cytokines, such as IFN-γ, that activates macrophages to kill the intracellular *Leishmania* parasites (Howard, 1986; Cummings *et al*., 2010). In this study SLA was prepared by two different methods, and denoted as SLA1 and SLA2. The presence of gp63 as a marker in SLA preparations was determined by western-blotting using rabbit anti *L. mexicana* gp63 antibodies. Glycoprotein gp63 is a main surface glycoprotein expressed on amastigotes and promastigotes of all *Leishmania* species (Yang *et al*., 1990; Yao, 2010). It has an important role in attachment, successful presentation of *Leishmania* parasite on MHC class II molecule and it has been shown to be capable of CD4+ T cell activation in visceral *Leishmania* infection and the potential as a protective immunogen in mice (Prina *et al*., 2004). A greater concentration of gp63 protein was detected in SLA2 compared with SLA1. The immunogenicity of SLA1 and SLA2 was assessed in protection experiments against *Leishmania* infection *in vivo* and cellular immune responses were analysed *in vitro* by immunological assays. It was shown that mice immunised with SLA1 or SLA2 were significantly protected against challenge with *L. mexicana* where (4 out of 6) mice, whether immunised with SLA1 or SLA2, remained free of lesion (Fig 3.2.4-A&B). Similar studies have shown that Balb/c mice immunised intranasally (I.N.) with *Leishmania*-derived recombinant polyprotein (Leish-111f) plus cholera toxin (CT) as adjuvant 1 to 3 times, induced significant protection against challenge with 10×10^6 of *L. major* promastigotes two weeks after the last immunistion. Also, splenocytes from I.N. immunised mice produced high levels of IFN-γ but not IL-4 in response to Leish-111f (Sakai *et al*., 2010). In addition, Trigo *et al*., (2010) studied the possible

immunotherapeutic efficiency of the subunit vaccine Leish-111f + monophosphoryl lipid A in stable emulsion (MPL-SE), which has undergone rigorous preclinical testing and been demonstrated safe in human clinical trials. Two separate trials were performed in Brazil and Salvador to evaluate the vaccine for therapeutic efficacy against CVL caused by natural infection: an Open Trial and a Blinded Trial. In the Open Trial 59 dogs with clinically active CVL have four groups: the first group: Leish-111f + MPL-SE, group two: treated with Glucantime, group three: a mixture of the vaccine and Glucantime, and final group: no treatment as control after six-month the 13 non-treated dogs had died or showed no clinical development. In contrast, nearly all dogs in groups 1 to 3 showed initial improvement (100%, 80%, and 92%, respectively).

In this study, it was found that mice immunised with SLA mixed with IFA, but not with DCs loaded with SLA, were significantly protected against challenge with live *L. mexicana* parasites, these experiments have also showed that IFA adjuvant alone did not stimulate specific anti SLA responses since mixing IFA with poor immunogenic ALS fractions did not induce significant responses (Fig 3.2.9). Nashed *et al*., (2000) have shown that IFA adjuvants were necessary to enhance immune responses when combined with Ags but on their own did not induce specific immune response. Surprisingly *Leishmania* lesions in mice immunised with DCs alone (normal DCs) showed a delayed appearance of lesions in comparison to control mice or mice immunised with DCs loaded with SLA. Different DC-based vaccination strategies against a number of diseases have been used. Tumour antigen pulsed DCs or DCs prepared to secrete cytokines (for example IL-12 or IL-18) are able to generate anti-tumour immunity (Tatsumi *et al*., 2003). Furthermore, DC vaccination has been used to promote immunity to infectious diseases; DCs are long-lived and

can ensure the maintenance of an efficient level of stimulation for T cells in draining lymph nodes (Mbow *et al*., 2001).

In this study it was found that, Balb/c mice immunised with DCs loaded with *L. mexicana* SLA1 or SLA2 induced specific CTL activity, but it was at a lower level compared with mice immunised with SLA in IFA, against DCs loaded with corresponding SLA. Ahuja *et al*., (1999) have shown that DCs secreting IL-12 and pulsed with soluble *L. donovani* antigens (SLA) in *vitro* provided a potent vaccine in a Balb/c mouse model of *L. donovani* infection. Antigen-pulsed as well as *Leishmania*-infected DCs were used for vaccination and was shown to be effective. In another study, DCs infected with *L. major* protected Balb/c mice against challenge with *L. major* (von Stebut *et al*., 2000) and DCs used as natural adjuvant also induced protective immunity against Leishmaniasis in this mouse model (Flohe *et al*., 1998; McKee *et al*., 2010). Moreover, DCs loaded with a combination of the recombinant *Leishmania* antigens gp63, LACK, PSA and KMP-11 or with the single antigen LeIF induced significant protection against challenge with *L. major* parasites (Berberich *et al*., 2003). Scott *et al*., (1987a). Studies have shown that CD8+ T cells were essential for resistance to reinfection in Balb/c mice (da Conceicao-Silva *et al*., 1994; Titus *et al*., 1987). Ravindran *et al*., (2010) have compared the effect of two different adjuvants, Bacille Calmette-Guerin (BCG) and Monophosphoryl lipid A (MPL) plus trehalose dicorynomycolate (TDM) with cationic liposomes, mixed with *L. donovani* promastigote antigens LAg against Balb/c mice visceral Leishmaniasis. All the three vaccines induced significant protection against *L. donovani* in the visceral organs, liver and spleen. Significant increase in IgG levels were detected in both MPL-TDM+LAg and liposomal Lag immunised animals with higher levels of IgG2a than IgG1.The highest level of protection was shown in the liposomal LAg immunised

group. Assessing immune responses by vaccination stresses the need of stimulation of strong cellular immunity that based on both Th1 and Th2 cells responses to confer protection against visceral Leishmaniasis.

In the present study potent CTL activity was demonstrated in splenocytes from Balb/c mice immunised with *L. mexicana* SLA2 and SLA2 fractions in combination with IFA against DCs targets loaded with corresponding SLA fraction, irrespective of the presence or absence of detectable gp63. Therefore, this suggests that SLA contains other proteins which could induce potent $CD8^+$ cytotoxic T lymphocyte activity. However only the whole SLA2 was capable of inducing the high levels of CTL compared with the SLA fractions. Carrillo *et al*., (2007) showed that immunisation with SLA produced an up-regulation of the IFN-γ mRNA in peripheral blood mononuclear cell (PBMC) from asymptomatic animals and Yamakami *et al*., (2001) reported that the co-administration of an IL-12 plasmid construct and SLA could prevent the development of lesions in the footpad of susceptible Balb/c mice. The protective effect of SLA immunisation was due to the development of a Th1 response. In addition, Balb/c mice immunised with A-SLA (Amastigote Soluble *Leishmania* Antigen) in combination with IFA followed by injection of 1×10^6 live promastigotes into the footpad, induced long-term protection from Leishmaniasis (Rafati *et al*., 2000). Scott *et al*., (1987b) separated SLA from *L. major* into nine distinct fractions by anion exchange liquid chromatography, and showed that only two fractions (one and nine) stimulated lymphocytes to produce macrophage-activating factor and elicited significant delayed-type hypersensitivity *in vivo*.

Nico *et al*., (2009) have shown that, Nucleoside Hydrolase (NH36) is the main marker of the fucose mannose ligand (FML) complex of *L. donovani*. The main epitopes of the NH36 recognized by MHC class I and II controlled T cells were the

sequences of three fragments composed by the amino acids 1-103 (F1), 104-198 (F2) and 199-314 (F3) in the pET28b plasmid. Balb/c mice vaccinated with NH36 recombinant protein and challenged with *L. chagasi* amastigotes induced significantly response to *Leishmania* antigen. F1 and F3 also induced a significant higher level of IFN-γ and TNF-α in spleen cells culture.

In another study by Rafati *et al*., (1997) SLA from both developmental stages of *L. major* was investigated, where sub-fractions three and five of SLA from the amastigote and promastigote stages were obtained by FPLC. Biochemical analyses revealed that the first fraction of amastigotes of *L. major* possessed a separate band following electrophoresis, corresponding to 24 KD, which induced a strong immune response to *L. major* compared with the other fractions. Rafati *et al*., (2000) reported the purification of a stage specific antigen from Amastigote Soluble *Leishmania* Antigen (A-SLA) of *L. major* by immuno-affinity chromatography. The purified protein was characterized as a cysteine proteinase, named as Amastigote Cysteine Proteinase (ACP). Balb/c mice were immunised by two intraperitoneal vaccinations, at a month interval, with 5μg of ACP or A-SLA in Freund's complete adjuvant (FCA), and challenged four weeks later with $1x10^6$ *L. major* promastigotes. The immunised mice showed significantly fewer and smaller lesions compared with controls.

Iniesta *et al*., (2008) have shown that a humoral response occurs in *L. major* infected C57BL/6 and Balb/c mice against three *Leishmania* antigens: soluble *Leishmania* antigens (SLA), a kinetoplastic membrane protein (Kmp-11) and a chimeric recombinant protein formed by the genetic fusion of four cytoplasmic proteins (PQ). The results showed a wide difference in the recognition of SLA, Kmp-11 and PQ by the sera of both strains. The anti-SLA response of Balb/c mice was 100 times greater

than that of C57BL/6 mice. Antibodies against the recombinant Kmp-11 were detected only in infected Balb/c during the first stage of the infection, but a response to the PQ antigen was detected in a late lesion period. Moreover, Ramirez *et al*., (2010) demonstrated that vaccination with ribosomal protein extracts (from *L. major*) administered in combination with CpG oligodeoxynucleotides protected Balb/c mice against primary *L. major* infection and induced the long-term immunity to secondary infection. Soluble *L. major* exo-antigens (LmSEAgs) are potential candidates for vaccination against Leishmaniasis, as evaluated in Balb/c mice and human PBMC. Lymphoid cells from the mice immunised against infection with *L. major* proliferated when restimulated with LmSEAgs and produced interferon-γ and IL-4. In addition, LmSEAgs stimulated human peripheral blood mononuclear cells to produce large amounts of IFN-γ and some IL-5. These findings suggest that LmSEAgs may be a vaccine candidate for Leishmaniasis in humans (Tonui & Titust, 2006).

In this part of the study it was found that Balb/c mice immunised with SLA1&2 and SLA2 fractions induced an immune response against *L. mexicana in vivo* and *in vitro* which confirms and expands the previous findings.

Chapter 4 Result

Characterisation of the immune response to *L. mexicana* antigens

4.1 Introduction

Dendritic cells play an essential role in conferring resistance or susceptibility to *Leishmania* by driving the differentiation and proliferation of CD4+ T helper cells to either Th1 or Th2 subsets (von Stebut *et al*., 2000). On presentation of *Leishmania* antigens to CD4+ T cells, the induction of IL-12 drives the proliferation of IFN-γ secreting Th1 cells and NK cells which activate macrophages and inhibit Th2 responses (Ruiz & Becker, 2007). In contrast the secretion of IL-4 during antigen presentation to CD4+ T cells drives Th2 cell development which inhibits Th1 responses and promotes B lymphocyte growth and development (von Stebut & Udey, 2004; Ueno *et al*., 2010).

DCs are also capable of inducing the clonal expansion of T helper cells; they are unable to induce T cell differentiation towards Th1 or Th2 without IL-12 and IL-4, respectively (Macatonia *et al*., 1993; Watchmaker *et al*., 2010) which confirms the importance of these cytokines in the immune response to *Leishmania*. Remarkably, the roles of IL-12 and IL-4 in antagonistic Th1 and Th2 responses were uncovered based upon observations with *L. major* (Alexander & Bryson, 2005; Murray *et al*., 2005). The protecting role of IL-12 in Leishmaniasis has been more or less well established. In a study by Mattner *et al*., (1997) IL-12 knockout mice, originally derived from a strain genetically resistant to infection with *L. major* were shown to be susceptible to infection with this parasite. However, Vanloubbeeck & Jones, (2004) have found that promoting Th1 polarisation of CD4+ T cells, using IL-12 as an adjuvant, was not sufficient to offer resistance to *L. amazonensis,* suggesting that no single cytokine alone could elicit protective immunity.

The role of IL-4 in disease progression has been suggested by several studies in which administration of anti-CD4+ and anti-IL-4 antibodies healed *Leishmania* infection (Awasthi *et al*., 2004). In addition, Kopf *et al*., (1996); Ehrchen *et al*., (2010) demonstrated that disruption of the IL-4 gene in susceptible Balb/c mice provided them with resistance to *L. major* infection; this result clearly reveals the effects of this cytokine on disease progression. Some studies recognized the role of IFN-γ in activating Th1 responses and resistance to *Leishmania* infection, while IL-4 is the major cytokine determining Th2 responses and disease susceptibility (Guimaraes *et al*., 2006). However, Noben-Trauth, (2000) has shown that IL-4 knockout Balb/c mice remained susceptible to *L. major* infection despite the absence of IL-4, indicating that *L. major* parasites may evade immune killing by different pathways other than IL-4. On the other hand, IL-2 is an essential cytokine for the production of IL-4 by CD4+ T cells and for the progression of Th2 responses *in vitro* and *in vivo* (Heinzel *et al*., 1993). Bryson *et al*., (2011) have shown that upon infection with *L. mexicana*, initial lesion in Balb/c mice is dependent on non-T cell populations receptive to IL-4/IL-13 whilst progressive infection is dependent on $CD4^+$ T cells receptive to IL-4. IL-2 also supports IFN-γ production and was shown to be important along with IFN-γ and IL-4 in the immunopathology of progressive Leishmaniasis (Sadick *et al*., 1990). In a previous study two different vaccines have been used evaluate the influence of IL-10 production on the quality, magnitude and protective ability of CD4+ T cell responses to *L. major* infection in mice. Multi-parameter flow cytometry was used to define CD4+ T cell production of IFN-γ, IL-2, TNF-α and IL-10 after vaccination. Mice immunised with a high dose of adenovirus (ADV) expressing *Leishmania* polyprotein (MML-ADV) had a low frequency of multifunctional IFN-γ + IL-2+ TNF + Th1 cells and a high frequency of IL-10

producing CD4+ T cells but, were not protected against challenge with live parasites. However, in the absence of IL-10, there were no changes in the magnitude, quality, or protective ability of the Th1 response obtained by high dose of MML-ADV. In contrast, mice immunised with MML + CpG, IL-10 down regulated the production of IL-12 by DCs *in vivo*, thus decreasing the generation of multifunctional Th1 cells. Consequently, three immunisations with MML + CpG were required for full protection. However, inhibiting IL-10 at the time of immunisation improved the magnitude and quality of the Th1 response sufficiently to mediate protection after only a single immunisation (Darrah *et al*., 2010). Furthermore, Zhoua *et al*., (2010) demonstrated that IL-10 aided the progression of cutaneous Leishmaniasis and suppression of the asthma allergic responses. Hepatitis B core Antigen (HBcAg) was used as a carrier to develop IL-10 peptide based vaccine for the control of IL-10 related diseases. The vaccine was designed by inserting a peptide from mouse IL-10 into the carrier molecules (HBcAg) using gene recombination methods. The vaccine, however, failed to protect against Leishmanisis because at least in this model the IL-10 vaccine enhanced the bioactivity of IL-10.

In a study by Rafati *et al*., (1997) sub fractions from a *L. major* amastigote antigen preparation were isolated and tested for induction of proliferation of IFN-γ and IL-4 production in cultures of PBMC from patients with *L. major* cutaneous Leishmaniasis. IFN-γ, but not IL-4, was significantly produced in response to stimulation with the first fraction of *L. major* amastigote. A *Leishmania* amastigote cysteine proteinase (ACP) was detected in the first fraction of the *L. major* amastigote-SLA. ACP, with an apparent molecular weight of 24 KD, was identified as a potential protective antigen against *Leishmania major*. The components of this fraction were also shown to induce a high level of IFN-γ which induces protection

against cutaneous Leishmaniasis (Rafati *et al*., 1997). Rafati *et al*., (2000) have also shown that Balb/c mice immunised with whole amastigote soluble *Leishmania* antigen A-SLA or purified ACP alone or in combination with Freund's adjuvant, induced long-term protection from Leishmaniasis as evaluated by reduced footpad swelling. Furthermore stimulation of splenocytes from mice immunised with purified ACP induced significant levels of cell proliferation and IFN-γ production.

In this study lymphocyte proliferation was used to investigate the splenocyte response from either naïve mice or mice immunised with *L. mexicana* SLA2 or six individual SLA2 sub-fractions separated by anion exchange Mono Q HR 5/5 column. An attempt was also made to determine the type of immune response (Th1 versus Th2) by measuring the levels and the type of antibodies produced in *Leishmania* sensitive Balb/c mice immunised with SLA1 and SLA2. Furthermore ELISA assay was used to measure the levels of IL-2, IL-4, IL-12 and IFN-γ in splenocyte cultures of Balb/c mice immunised with SLA2 and SLA2 fractions, the summarizing of experimental work in this chapter shown in (Fig 4.1).

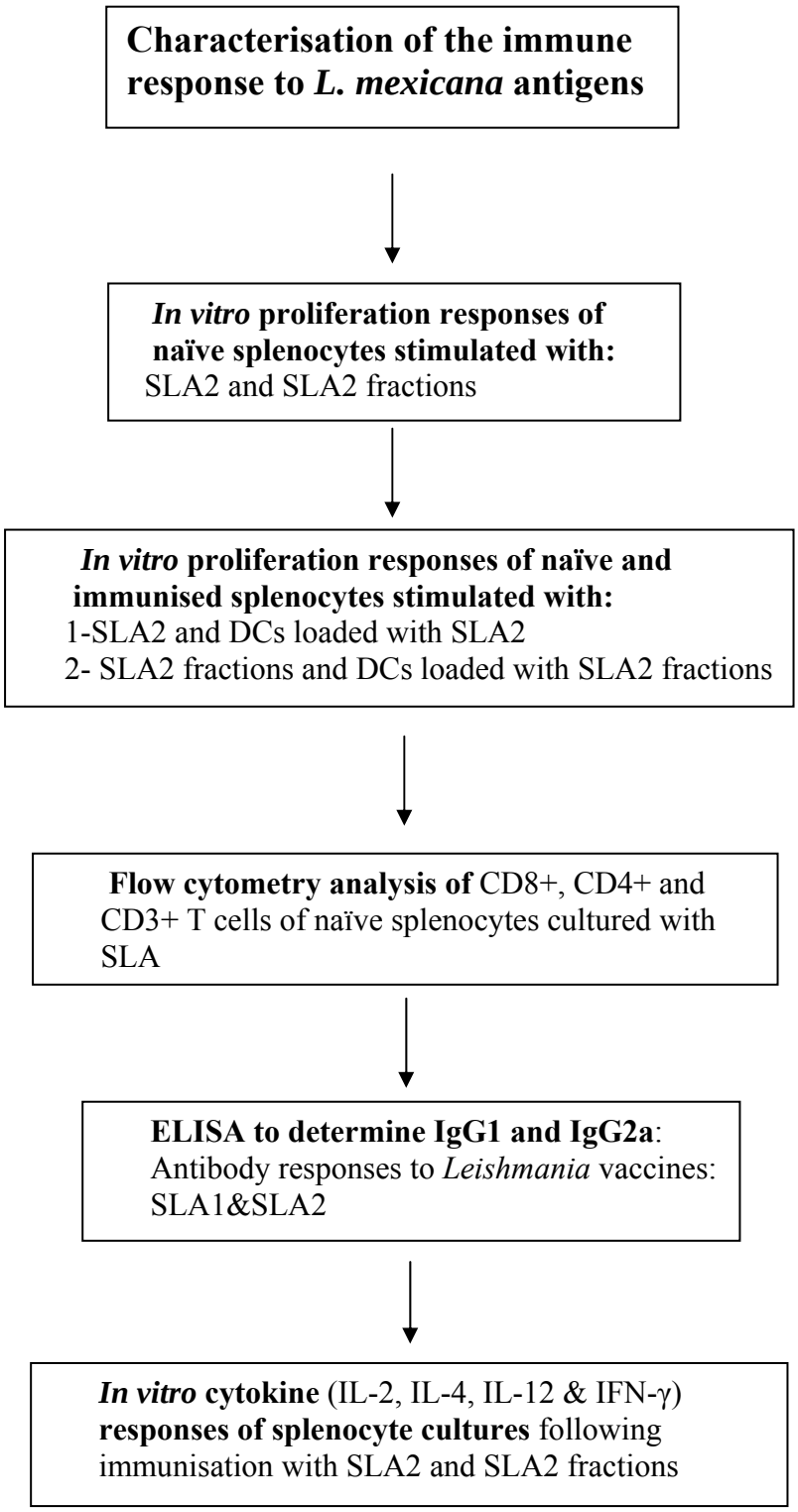


Figure 4.1 A flow chart summarizing the experimental work in this chapter.

4.2 Results

4.2.1 Proliferation responses of splenocytes stimulated with SLA2 and SLA2 fractions

In the present study the proliferation responses of splenocytes from naïve and immunised Balb/c mice to stimulation with SLA2 and SLA2 fractions alone or loaded on to DCs was investigated using *in vitro* tritiated thymidine uptake proliferation and cytokine assays.

4.2.1.1 *In vitro* proliferation responses of naïve splenocytes to stimulation with SLA2 and SLA2 fractions.

In this study the proliferation of splenocytes derived from naïve mice stimulated with SLA2 and SLA2 fractions was investigated. Naïve splenocytes of Balb/c mice were prepared as described on (chapter 2 methods). The results (Fig 4.2.1.1) show that stimulation of naïve splenocytes of Balb/c mice with SLA2 and SLA2 fractions (apart from fr4 and fr6) induced a significant proliferation response, after 7 days of culturing with *Leishmania* antigens. The proliferation response nearly doubled when splenocytes were cultured with antigens for 14 days when even fr4 & fr6 induced a significant proliferation response.

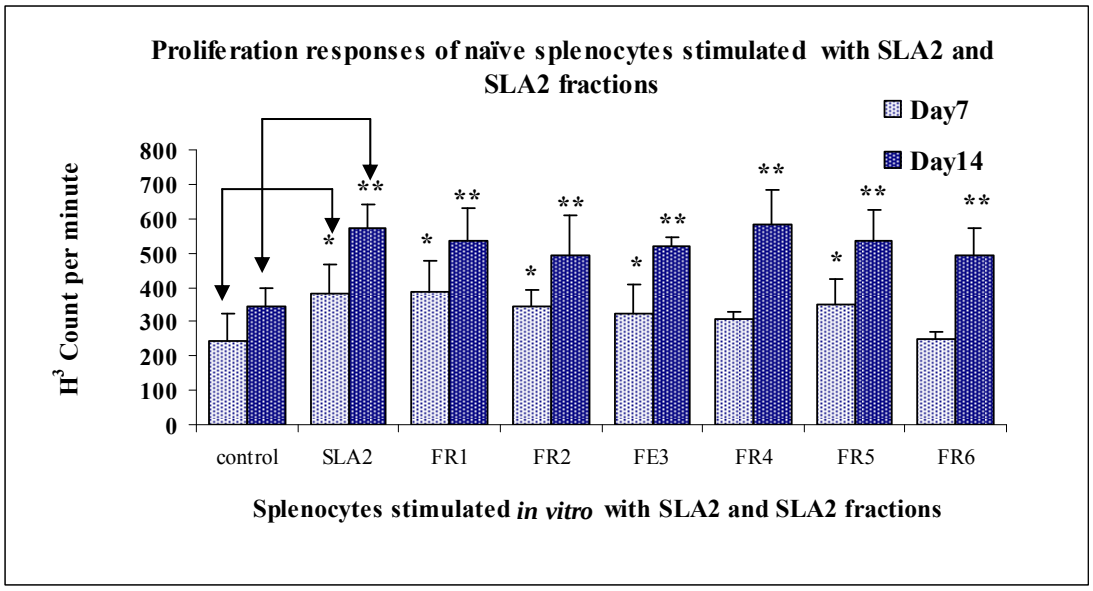


Figure 4.2.1.1 Proliferation of naïve splenocytes stimulated with SLA2 or SLA2 fractions (fr1-6) Naïve splenocytes of Balb/c mouse were flushed out from the spleens by serum-free RPMI 1640 media. The spleen cells were washed and resuspended in CTL media. Cells were counted and plated at 5×10^4/100µl CTL media per well, with 10µg/ml of whole SLA2 or SLA2 fractions (fr1, fr2, fr3, fr4, fr5 and fr6). Cells were incubated at 37°C for 7 and 14 days, control was naïve splenocytes with media. ^{3}H was added to each well 18 hours before the termination of the assay. Bars represent the standard deviation of the mean n=6 p*≤0.05, p**≤0.01 and p***≤0.001 by T test.

4.2.1.2 Proliferation responses of naïve and immunised Balb/c mice splenocytes to stimulation with DCs loaded with SLA2 and SLA2 fractions

The proliferation responses of naïve and SLA2 immunised mice to DCs loaded with corresponding SLA2 or SLA2 fractions were investigated. DCs were generated as previously described (chapter 2 methods). Naïve and immunised splenocytes were divided into four groups and stimulated with each antigen (SLA2 or SLA2 fractions): (1) splenocytes stimulated with SLA2 or SLA2 fractions (fr1-6), (2) splenocytes stimulated with DCs loaded with SLA2 or SLA2 fractions (3) splenocytes stimulated with DCs alone (as control) and (4) splenocytes cultured in media. The protocol for stimulations is shown in Table 4.2.1.2. ^{3}H was added 18 hours before harvesting cells on days 7 and 14.

Splenocytes source	**Stimulated with**			
naïve mouse	DCs alone	DCs +SLA2	SLA2	media
naïve	DCs	DCs +fr1	Fr1	media
naïve	DCs	DCs +fr2	Fr2	media
naïve	DCs	DCs +fr3	Fr3	media
naïve	DCs	DCs +fr4	Fr4	media
naïve	DCs	DCs +fr5	Fr5	media
naïve	DCs	DCs +fr6	Fr6	media
Immunised mouse	**DCs**	**DCs +SLA2**	**SLA2**	**media**
Immunised	DCs	DCs +fr1	Fr1	media
Immunised	DCs	DCs +fr2	Fr2	media
Immunised	DCs	DCs +fr3	Fr3	media
Immunised	DCs	DCs +fr4	Fr4	media
Immunised	DCs	DCs +fr5	Fr5	media
Immunised	DCs	DCs +fr6	Fr6	media

Table 4.2.1.2 Stimulation of Splenocytes with DCs loaded with SLA Ags
Splenocytes were either obtained from naïve or immunised Balb/c mice and cultured with the corresponding SLA2 or SLA2 fractions. Mice were immunised 7 days before the start of proliferation assay with corresponding antigens.

Naïve and immunised splenocytes were stimulated with SLA2, fr1, fr2 or DCs loaded with SLA2, fr1 or fr2 as described above. The results show that stimulation of naïve splenocytes *in vitro* with SLA2, fr1 and fr2 or DCs loaded with SLA2, fr1 and fr2 induced significant proliferation following 14 days incubation compared with 7 days. However, stimulation of splenocytes from immunised mice with SLA2, fr1 and fr2 or DCs loaded with SLA2, fr1 and fr2 induced significant proliferation following 7 and 14 days incubation compared with naïve splenocytes (Fig 4.2.1.2.A&B).

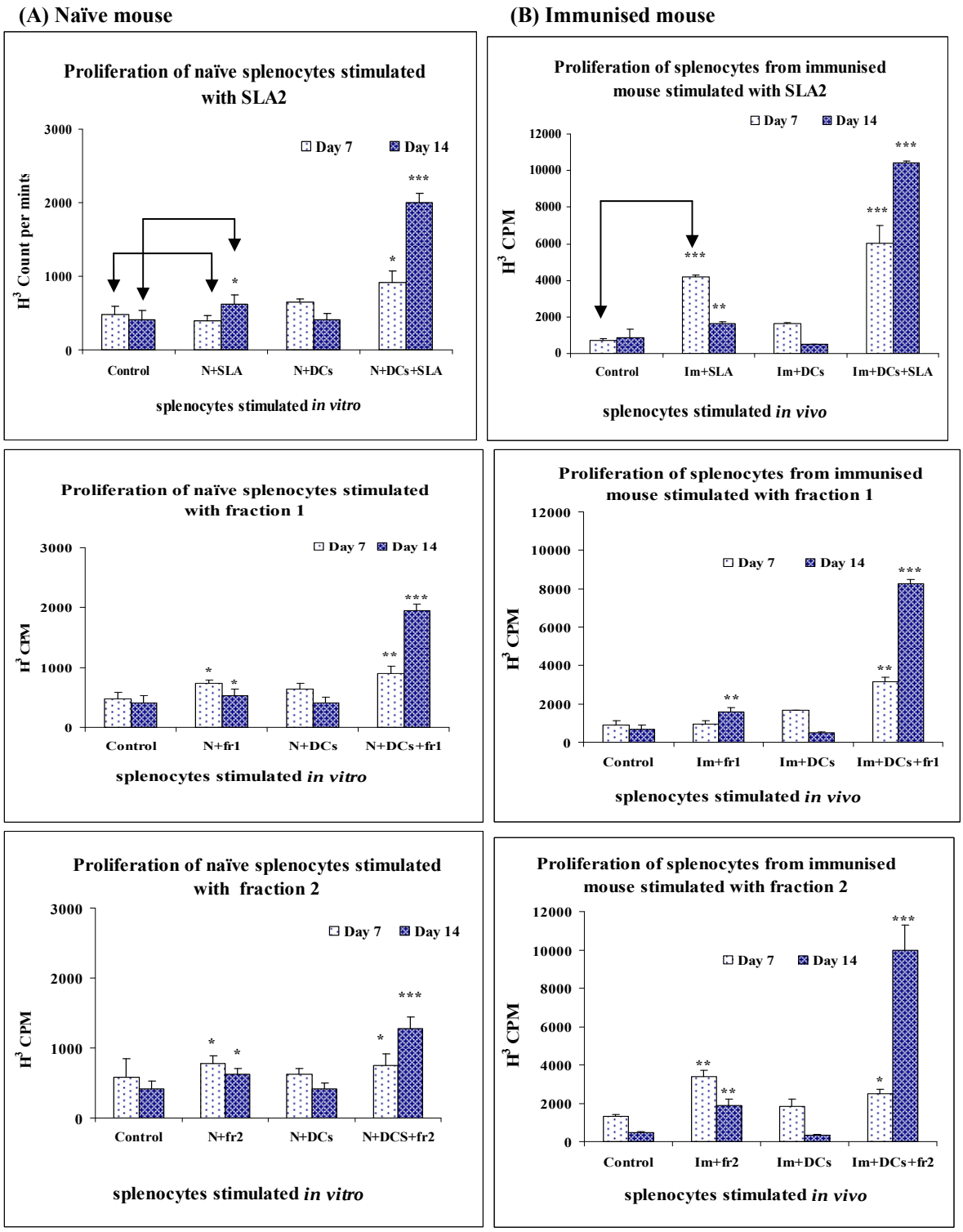


Figure 4.2.1.2 Proliferation response of naïve (A) and immunised (B) splenocytes stimulated with SLA2, fr1 & fr 2 or DCs loaded with SLA2 and DCs loaded with (fr1&2) respectively
BMDC's cells were cultured in the presence of GM-CSF for 6 days with washes every 2 days. On day 6 DCs were pulsed with 10µg/ml SLA2, Fr1 and fr2 and then pulsed 4-6 hours later by 1µg/ml LPS then incubated overnight. On day 7, DC cells were pulsed again with 10µg/ml SLA2 or other antigens for 1hr and were used to stimulate splenocytes. Splenocytes were harvested one week after immunisation or from a naïve mouse and plated into 96 well plates at 5×10^4cells/well. Cells were then incubated with10µg/ml SLA2 or fr1&2 and DCs+SLA2, DCs+ fr1 and DCs+ fr2 or DCs alone or media as controls. ^{3}H was added 18 hours before harvesting the cells on days 7 and 14. Bars represent the standard deviation of the mean n=6 p*≤0.05, p**≤0.01 and p***≤0.001 by T test.

Balb/c mice were immunised with SLA2-fr3, fr4, fr5 or fr6 mixed with IFA seven days prior to the proliferation assay. Naïve and immune splenocytes were isolated as previously described and stimulated with fr3, fr4, fr5 or fr6 or DCs loaded with fr3, fr4, fr5 or fr6 (section 4.2.1.2). Results in (Fig 4.2.1.2.1) clearly show that similar proliferation was observed by all fr3, fr4, fr5 and fr6 in naïve and immunised mice for 7 and 14 days cultures and significant but low proliferation responses were observed for these fractions.

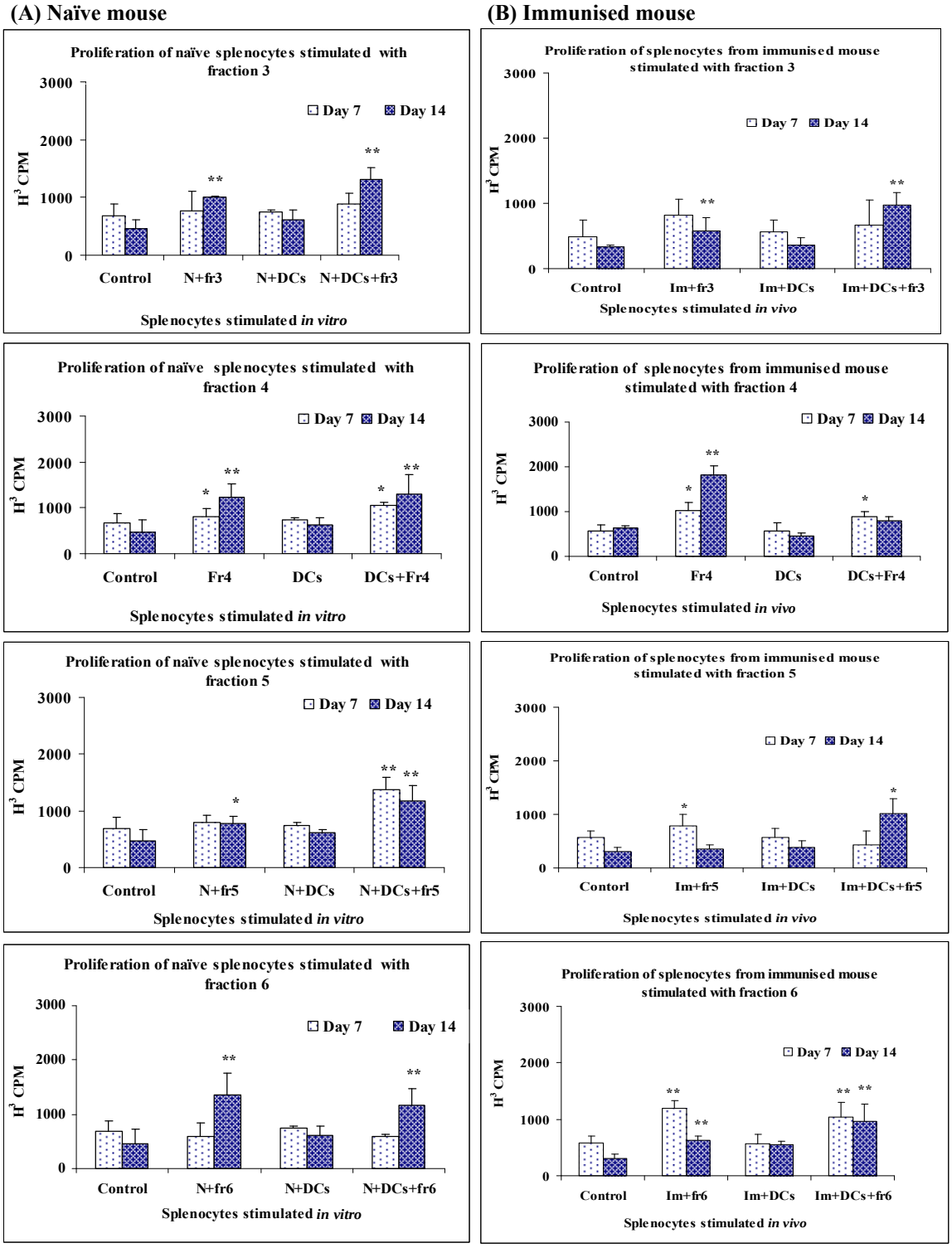


Figure 4.2.1.2.1 Proliferation response of naïve (A) and immunised (B) splenocytes stimulated with DCs loaded with fr3-6 respectively

BM-DC cells were cultured with GM-CSF for six days and then washed every two days. On day six, DCs were pulsed with 10µg/ml fr3-fr6 and after 4-6 hours pulsed with 1µg/ml LPS and incubated for overnight. On day seven, DC cells were pulsed again with 10µg/ml antigen for 1hr and were used to stimulate splenocytes. On day 7 of DCs, splenocytes from naïve or immunised mice with SLA2-fr3, fr4, fr5 or fr6 mixed with IFA for 7 days, were divided into four groups and stimulated with DCs pulsed with fr3-6 or media. DCs alone were used as control. ^{3}H was added 18 hours before harvesting the cells on days 7 and 14. Bars represent the standard deviation of the mean n=6 p*≤0.05, p**≤0.01 and p***≤0.001 by T test.

4.2.2 Flow cytometry analysis of CD8+, CD4+ and CD3+ T cells of naïve splenocytes cultured with SLA

In this study the effect of stimulation with SLA2 on the expansion of CD4+ and CD8+ T cells of naïve splenocytes using CD4+, CD8+ and CD3 markers was investigated. Splenocytes of Balb/c mice were flushed from the spleens using serum-free RPMI 1640 media (see chapter 2 methods) splenocytes were stained with FITC labelled anti CD3, CD4, CD8, and IgG2a (isotype control) as a negative control and analysed by flow cytometry (Table 2.10). The results clearly show an expansion of CD4+ and CD8+ T cells as indicated by the increase in the frequency of CD4, CD8 and CD3 markers compared with control groups (Fig 4.2.2), suggesting T-cell subset expansion. The expression of CD3 on control group was less than that of CD8 and CD4; it may be due to the poor quality of the antibody used.

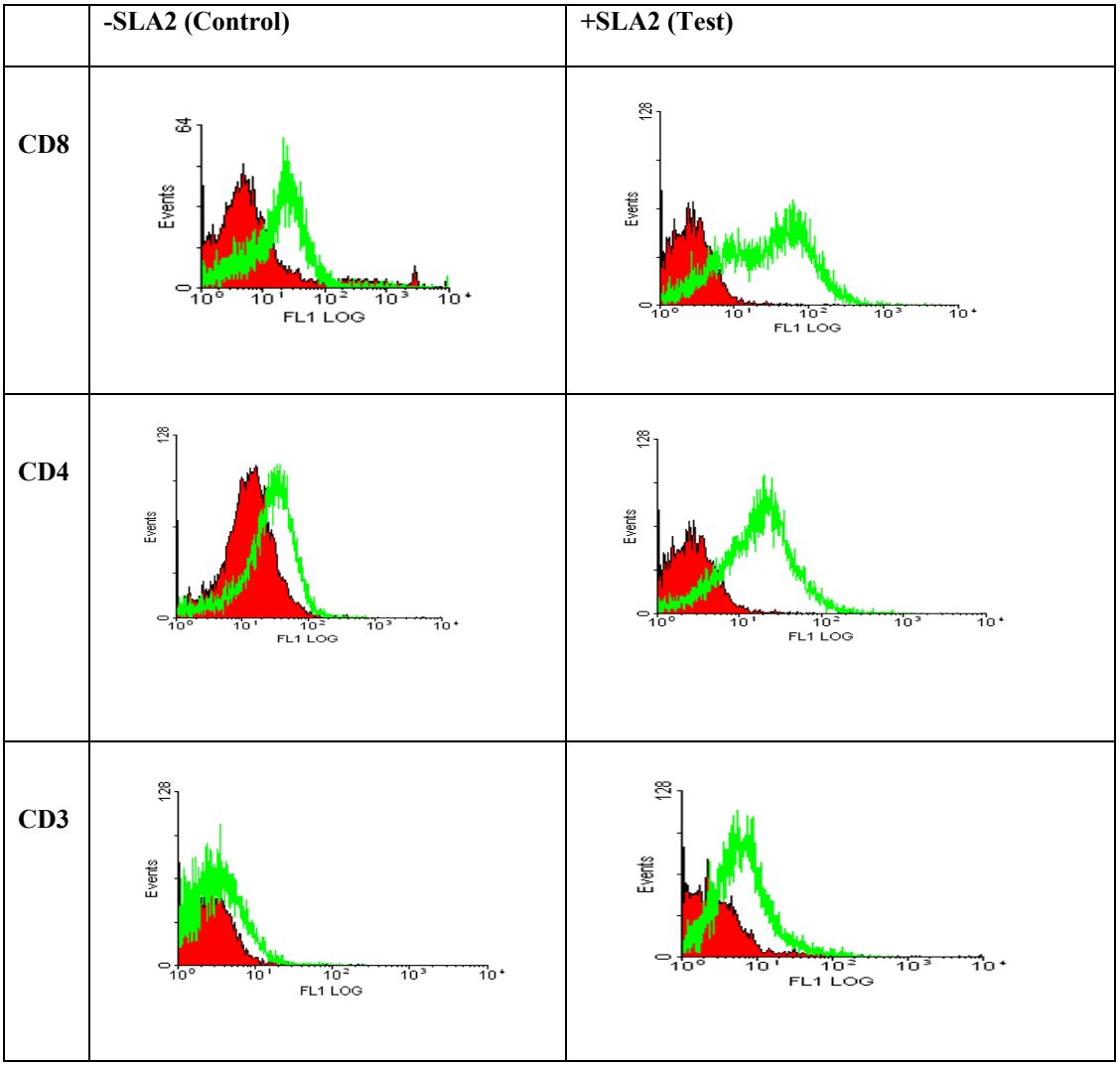


Figure 4.2.2 The expansion of CD8 + and CD4 + T cells and CD3 following stimulation with SLA2
Splenocytes were flushed out from the spleens by serum-free media. The spleen cells were collected, washed and resuspended in CTL media. Splenocytes were divided into two groups at 5×10^6/well/ml: the first group was used as control and the second group was stimulated with SLA2. On day 5 each group was divided into four at 2×10^5 cells per tube, and stained with anti CD3, CD4, CD8, and IgG2a as a negative control and analysed by flow cytometry. The graph represents 3 independent experiments. Red: control, green: test.

4.2.3 Antibody responses to *Leishmania* vaccines

Th1/Th2-type immune responses were assessed by measuring the level and the type of antibodies in immunised *Leishmania* sensitive Balb/c mice. Mice were immunised S.C. at the base of the tail either with 100µg/mouse of SLA1 or SLA2 and then bled 4 times at weekly intervals and the level of anti-*Leishmania* IgG1 and IgG2a isotype

antibodies was determined by ELISA (see chapter 2 methods). The results clearly demonstrate an increase of IgG2a and IgG1 in the serum of mice immunised with SLA1 and SLA2 as early as 7 days after the immunisation. The antibody response against SLA2 in the first 2 weeks was higher than that against SLA1. Both IgG2a/IgG1 antibody responses were detected, thus demonstrating a complex Th1/Th2 immune response to SLA1 and SLA2 immunisation (Fig 4.2.3).

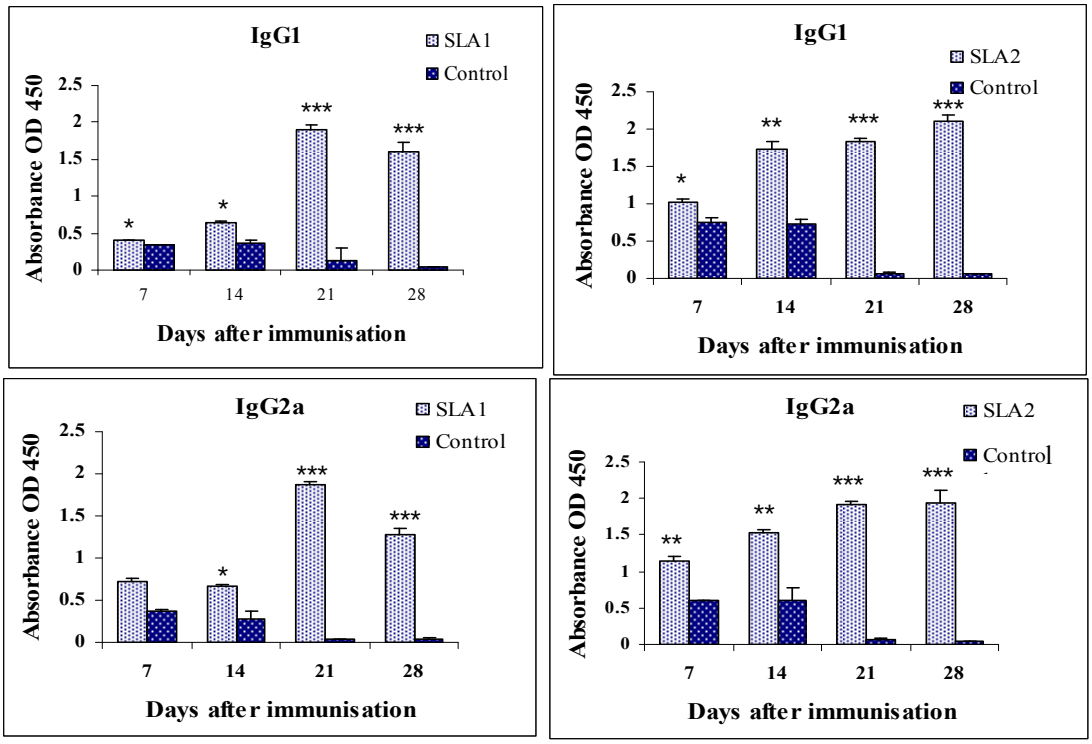


Figure 4.2.3 Antibody responses in mice immunised with SLA1 and SLA2
Balb/c mice were immunised S.C. at the tail base with either 100µg/mouse SLA1 or SLA2 mixed with IFA. Serum samples were collected after immunisation on days 7, 14, 21 and 28. Levels of IgG1 and IgG2a were determined by ELISA and corresponding SLA antigen and serum from naïve mouse was used as control. The graph represents 3 independent experiments n=6 p*≤0.05, p**≤0.01 and p***≤0.001 by T test.
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4.2.4 Production of IL-2, IL-4, IL-12 & IFN-γ following immunisation with SLA2 and SLA2 fractions

In this study, the production of cytokines following immunisation against *L. mexicana* in the Balb/c mouse model was investigated.

4.2.4.1 IL-2 Cytokine Production

IL-2 has been considered a key growth and death factor for antigen-activated T lymphocytes. IL-2 and IFN-γ have been shown to have important roles in the immune response and in the eradication of *Leishmania*. In order to analyse the T-cell responses in immunised and naïve Balb/c mice stimulated with SLA2 and SLA2 fractions, an ELISA was used to measure IL-2 levels in supernatants of splenocytes cultured *in vitro* with SLA2 and SLA2 fractions. Splenocytes were harvested from the spleens of naïve and immunised mice 7 days after immunisation (see chapter 2 methods). Supernatants were collected and analysed for IL-2 production using an ELISA assay. The results clearly demonstrated that stimulation of naïve and immunised splenocytes with the unfractioned SLA2 induced a greater amount of IL-2 than any single fraction, and that IL-2 release appeared to be greater for immunised splenocytes than a naïve mice (Fig 4.2.4.1A&B).

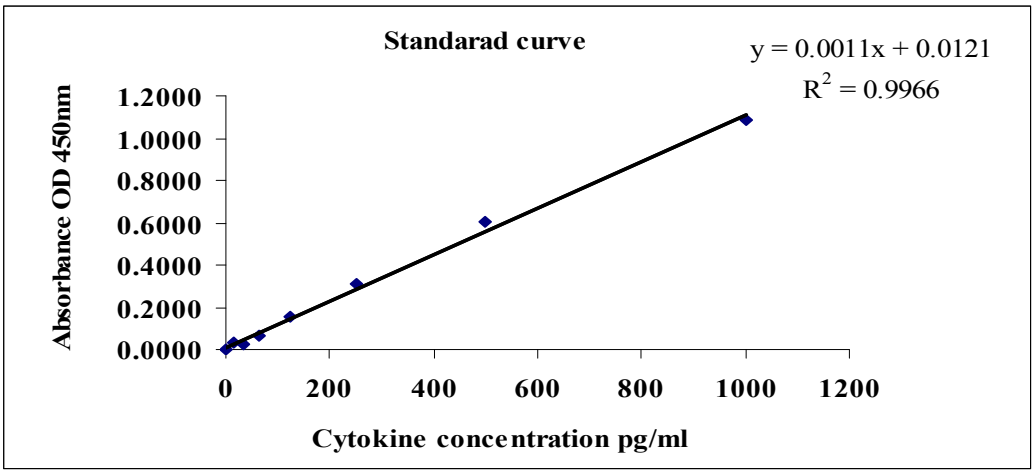


Figure 2.5.2 IL-2 cytokine standard curve. A standard curve was created to determine the level of cytokine by using known concentration.

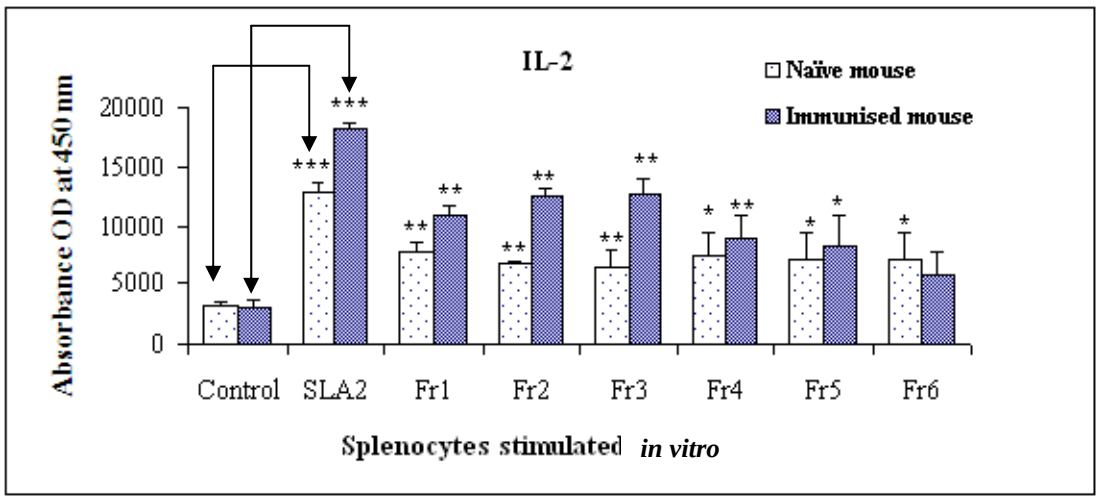


Figure 4.2.4.1-B IL-2 production by splenocytes from naïve and immunised Balb/c mice following stimulation with SLA2 and SLA2 fractions
Mice were immunised S.C. at the base of the tail either with 100µg/mouse of SLA2 or SLA2 fractions. Cultured splenocytes for 7 day with or without SLA2 and SLA2 fractions from immunised or naïve Balb/c mice were collected for cytokine measurement. Splenocytes from naïve mouse were used as control. The graph represents 3 independent experiments Bars represent the standard deviation of the mean n=6 p*≤0.05, p**≤0.01 and p***≤0.001 by T test.

4.2.4.2 IL-4 Cytokine Production

In this study the levels of IL-4 in supernatants of cultured splenocytes were determined by ELISAs. Splenocytes from naïve and immunised mice were collected and cultured as previously described in (see chapter 2 methods). The results (Fig 4.2.4.2), show that low but significant levels of IL-4 could be detected in supernatants of both naïve and immune splenocyte cultures in response to stimulation with whole SLA2, SLA2-fr4 and SLA2-fr5, but although this was shown to be statistically significant for fraction 4, 5 also SLA2, the levels never exceeded twice the control values.

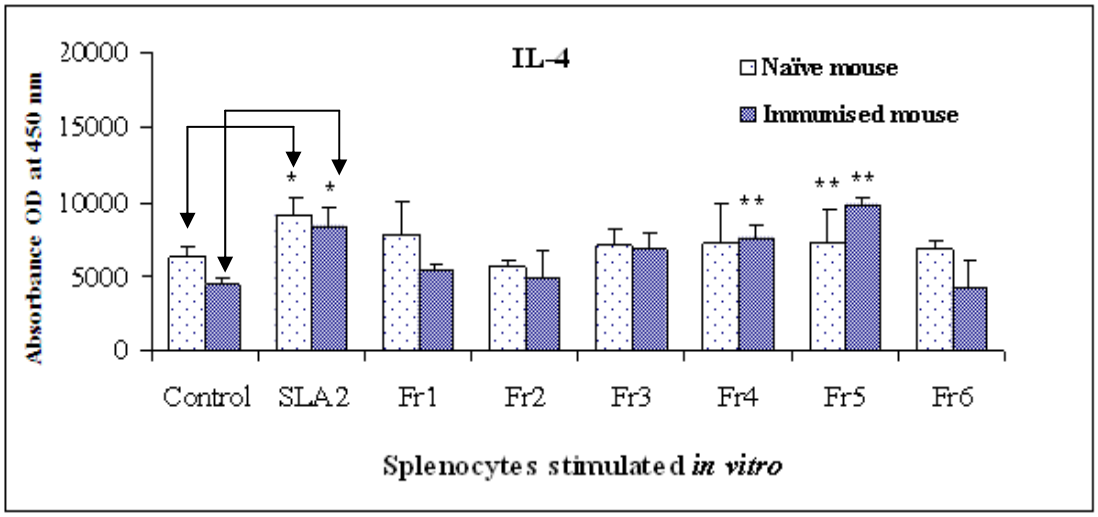


Figure 4.2.4.2 IL-4 production by splenocytes from naïve and immunised Balb/c mice
Splenocytes cultured for 7 days with SLA2 and SLA2 fractions or without SLA2 as control from immunised or naïve Balb/c mice were collected for cytokine measurement. The graph represents 3 independent experiments Bars represent the standard deviation of the mean n=6 p*≤0.05, p**≤0.01 by T test.

4.2.4.3 IL12-p70 Cytokine Production

Infection with *Leishmania* stimulates the production of IFN-γ, via a pathway which is dependent upon IL-12. IL-12 is also important for the progress of a host protective T cell response to this parasite. Mice were immunised with SLA2 and SLA2 fractions as described (see chapter 2 methods). Supernatants were assessed for IL-12-p70 release using ELISA. High levels of IL-12-p70 were detected in supernatants of both immunised and naïve Balb/c mice splenocytes with the highest levels present in cultures stimulated with whole SLA2. Interestingly naïve splenocytes stimulated with SLA2 or SLA2 fractions induced significant levels of IL-12-p70 (Fig 4.2.4.3).

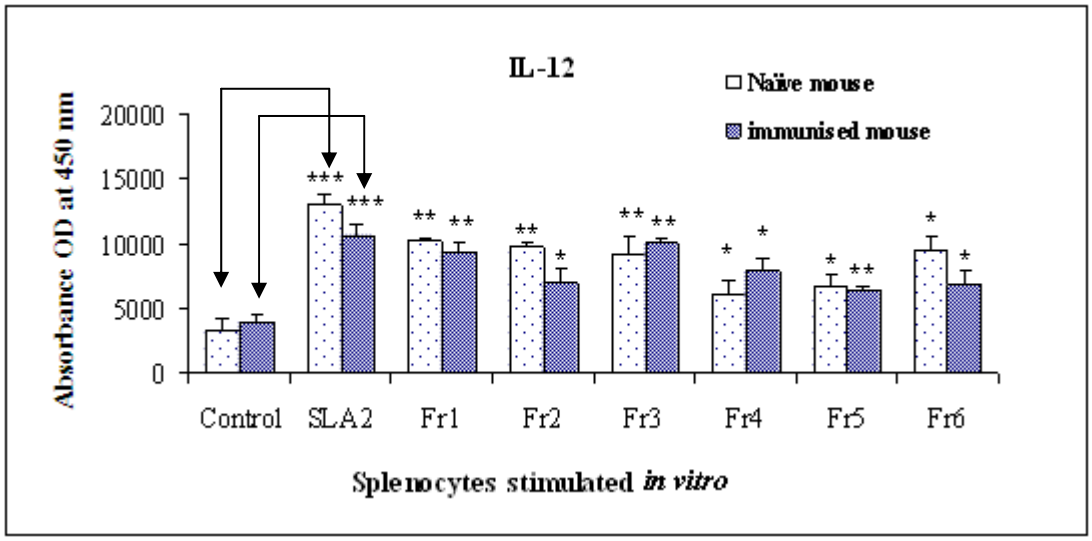


Figure 4.2.4.3 IL-12-p70 production by splenocytes from naïve and immunised mice
Splenocytes cultured for 7 days with or without SLA2 and SLA2 fractions of immunised or naïve Balb/c mice were collected and measured for IL-12. The graph represents 3 independent experiments. Bars represent the standard deviation of the mean n=6 p*≤0.05, p**≤0.01 and p***≤0.001 by T test.

4.2.4.4 IFN-γ Cytokine Production

IFN-γ plays an essential role in the activation of macrophages to kill intracellular parasites by inducing the production of nitric oxide. IFN-γ was detected by ELISA assay from supernatants of naïve and immunised splenocytes with or without SLA2 or SLA2 fractions. Splenocytes were stimulated (as described in section 4.2.4.1). The results clearly show that IFN-γ significantly increased in both naïve and immunised mice compared with the control (Fig 4.2.4.4).

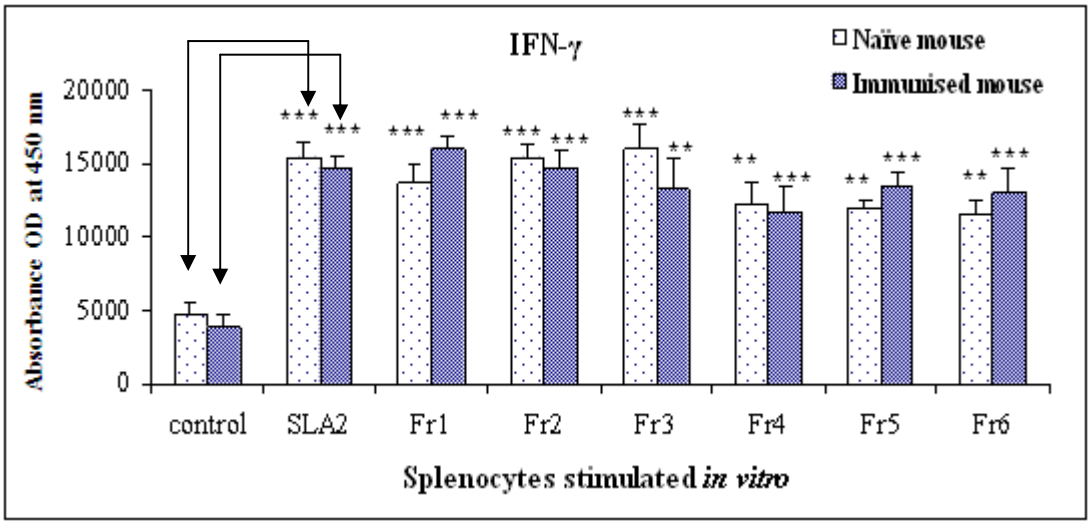


Figure 4.2.4.4 IFN-γ production by splenocytes from naïve and immunised mice stimulated with SLA2 and SLA2 fractions

Splenocytes from immunised and naïve Balb/c mice were stimulated with SLA2 or SLA2 fractions for 7 days. IFN-γ was assessed using an ELISA assay. The graph represents 3 independent experiments. Bars represent the standard deviation of the mean n=6 p*≤0.05, p**≤0.01 and p***≤0.001 by T test.

4.3 Discussion

An anti *Leishmania* immune response is dependent on the host's genotype. Studies using *Leishmania* mouse models have shown that some strains of mice are susceptible to Leishmaniasis, while others are resistant. Resistance of the host against parasitic infections is controlled by the activation and differentiation of CD4+ Th1 lymphocytes. In contrast, immune responses in susceptible mice are associated with activation and differentiation of Th2 (Handman, 2001; Bryan *et al*., 2010). IFN-γ secreted by Th1 cells, is a potent cytokine inducing macrophage activation, leading to host resistance to infection with *Leishmania* parasites, while IL-4 secreted by Th2 cells is associated with down modulation of IFN-γ macrophage activation (Ajdary *et al*., 2000). Scott *et al*., (1987b) found that I.P. injection of soluble, non-membrane fractions of *L. major* promastigotes SLA combined with the bacterial adjuvant *Corynebacterium parvum***,** protected Balb/c mice against infection with *L. major* as effectively as whole irradiated organisms. Inoculation with SLA was found to induce both humoral and cell mediated immune responses to *Leishmania* antigens. In this study *L. mexicana* SLA2 was separated into six SLA2 sub-fractions (fr1-6) by anion exchange Mono Q HR 5/5 column separation, and tested for their ability to cause lymphocyte splenocyte proliferation using splenocytes from naïve Balb/c mice or mice previously immunised with corresponding SLA2 or SLA2 fractions.

The results presented in this chapter clearly showed that *in vitro* stimulation of naïve Balb/c mouse splenocytes with SLA2 or SLA2 fractions induced a significant, but low proliferation response compared with higher responses induced by stimulation with DCs loaded with SLA2 or SLA2 fractions. The highest level of splenocyte proliferation was observed by stimulation with whole SLA2 and to a lesser extent

than with SLA2-fraction one and fraction two (Fig 4.2.1.2). In another study, SLA of amastigote and promastigote of *L. major* were fractionated by FPLC into 3 and 5 fractions respectively. The fractions were tested for proliferation responses, IFN-γ and IL-4 production by human PBMC cultures from patients with cutaneous Leishmaniasis. The first fraction of the amastigote SLA induced a higher proliferation response and IFN-γ but not IL-4 release, compared with the other fractions. Patients PBMC showed different proliferation response profiles to amastigote and promastigote SLA fractions; the promastigote SLA fractions induced a lower response compared to amastigote SLA fractions (Rafati *et al*., 1997). A response to SLA or SLA fractions seems to be strongly influenced by the *Leishmania* species used, the developmental stage and fractionation methods. Soluble *Leishmania* antigen (S-SLA) derived from highly infective stationary-phase *L. major* was fractionated by gel electrophoresis to isolate a low molecular mass fraction (<31 kDa) of S-SLA fraction D (FR D) which was found to strongly stimulate *L. major* specific Th1 helper cell clones (Bogdan *et al*., 1990). In addition, Nagill and Kaur (2010) have investigated the immune response to a 78 kDa antigen of *L. donovani* alone or with various adjuvants against mouse VL. The adjuvants used with the 78 kDa antigen were recombinant IL-12, monophosphoryl lipid A (MPL-A), autoclaved *Leishmania* antigen (ALA), liposomal encapsulation and Freund's adjuvant. Balb/c mice were immunised subcutaneously three times with the respective vaccine formulation. Significant protection against infection was obtained from the 78 kDa antigen + ALA and 78 kDa antigen + Freund's adjuvant but much less with the 78 kDa antigen alone.

It has been suggested that the outcome of infection is dependent on the activation pathway of one of the two subsets of CD4 T cells, Th1 or Th2. Th1-type cellular immune responses play a critical role in protection against infection with *Leishmania* parasites, but activation of Th2 type cells results in exacerbation of the disease (Ajdary *et al*., 2000; Cummings *et al*., 2010). ELISA was used to measure the levels of IL-2, IL-4, IL-12 and IFN-γ in Balb/c splenocyte cultures following immunisation with SLA antigens or infection with *L. mexicana*. Morris *et al*., (1994) have suggested that the type of antibody responses depend on the cytokines produced by antigen specific T cells, whereby IgG2a levels are regulated by IL-12 and IFN-γ, while IgG1 levels are controlled by IL-4. Two novel antigens (140 and 152 kDa) were extracted from soluble antigen of metacyclic promastigotes of *L. major* by western-blotting and found to induce specific IgG2a responses in Balb/c and C57BL/6 mice. The two antigens were also shown to be reactive to IgG antibody of cutaneous Leishmaniasis patients (Mohammadi *et al*., 2006). It has been reported in a study by Grimaldi & Tesh, (1993), that Th1 cells secrete IL-2 and IFN-γ when they come into contact with parasite antigens displayed on the macrophage membrane. IFN-γ can activate infected macrophages from both strains of mice (susceptible and resistant) to induce resistance to *L. major in vivo*.

T cells secreting IFN-γ undergo very slow proliferation against *in vitro* parasite antigens. IFN-γ along with IL-12, GMCSF and TNF-α activate macrophages which in turn express the iNOS enzyme, intracellular amastigotes are killed by the nitric oxide produced by iNOS. In fact IFN-γ is the only cytokine that can independently enhance iNOS transcription and release of nitric oxide from mouse peritoneal macrophages. IFN-γ also plays a major role in controlling *Leishmania donovani* infection (Squires

et al., 1989). In contrast Th2 cells secrete IL–10 and IL-4 which deactivates the macrophages, thus counteracting the action of Th1 cells. These cytokines promote the differentiation of Th2 cells in susceptible Balb/c mice and assist in disease progression (Awasthi *et al*., 2004). The results of this study demonstrated an expansion of CD4+ T cells as determined by *in vitro* stimulation with SLA2 and antibody staining for CD4, CD80 and T cell receptor (TCR)$^{-}$CD3 antigen. The cytokine response profile (discussed below) is indicative of a bias towards a Th1 response. Identifying new antigens with potential immunogenicity to activate Th1, rather than Th2 cells would be of a great value in designing new vaccines against *Leshismania* parasite. Rostami (2008) has found that *in vitro* stimulation with SLA for 7 days induced better responses in CD4+ and CD8+ lymphocytes isolated from human PBMC than live *L. major.*

In this *in vitro* study high levels of IL-2, IL-12 and IFN-γ but lower levels of IL-4 were detected in naïve and immunised Balb/c mice splenocyte culture supernatants stimulated with SLA2 or SLA2 fractions, suggesting a Th1 response. Resistance to *Leishmania* infection in C57BL/6 mouse strain is due to Th1 response as a result of the induction of these cytokines (Alfonso *et al*., 1994). The primary mechanism linked to the elimination of *Leishmania* is via the activation of macrophages by IFN-γ that are secreted by Th1, and NK cells which enable them to kill intracellular *Leishmania* amastigotes in a NO dependant manner (Stenger & Rollinghoff, 2001). Inhibiting IL-2 function by using blocking antibodies inhibits the production of IL-4, a cytokine normally associated with susceptibility to *Leishmania* infection by Balb/c mice. IL-2 therefore appears to be necessary for the expansion of Th2 CD4+ lymphocytes *in vivo* (Heinzel *et al*., 1993).

The role of IL-2 in the development of Th2 cells *in vitro* and *in vivo* in other disease models has been reported, and it is not clear whether the use of anti-IL-2 antibodies inhibit Th2 development directly by neutralising the activity or indirectly by inhibiting IL-4 production. CD4+ T cells from Balb/c mice infected with *L. major* release a wide range of cytokines including IL-2 and IFN-γ during the first week of infection (Reiner *et al*., 1994). In addition Rostami *et al*., (2010) have evaluated the immune response in Balb/c mice immunised three times with two different doses of Alum autoclaved *Leishmania major* (Alum-ALM), (50μg and 200μg), killed *Mycobacterium vaccae* (1×10^6 and 1×10^7) or one dose of BCG (1×10^7). Balb/c mice immunised with low dose of Alum-ALM mixed with any of low *M. vaccae* or BCG demonstrated a significantly high level of IFN-γ production and a low IL-4 level and a significantly lower parasite burden compared to the control PBS injected group. Also, immunisation with a low dose of Alum-ALM with an adjuvant induces a Th immune response in Balb/c mice. Collectively, these results suggest that immune response is influence by antigen type and immunisation regime.

In summary, *in vitro* and *in vivo* stimulation of naïve Balb/c mouse splenocytes with SLA2 or SLA2 fractions induced significant but low proliferation responses compared with higher responses induced by stimulation with DCs loaded with SLA2 or SLA2 fractions. Also high levels of IL-2, IL-12 and IFN-γ but less IL-4 were detected in naïve and immunised Balb/c splenocyte culture supernatant stimulated with SLA2 or SLA2 fractions, the level of IL-2, IL-12 and INF-γ of cytokines response detected infer a complex Th1 more than Th2 immune response following immunisation with SLA2 and SLA fractions.

Chapter 5 Result

Down-regulation of DC surface molecules is a possible immune evasion mechanism in *L. mexicana* infection

5.1 Introduction

Leishmania requires a number of immune-evasion mechanisms to resist phagolysosome fusion and prevent activation of more-potent acquired immune responses. The main adaptive strategies include the inhibition of IL-12 synthesis and induction of IL-10 and TGF-β by infected cells. These cytokines promote the shift of response from Th1 to Th2 (Taylor-Robinson, 2001). LPG and gp63 are responsible for the virulence of the parasite. LPG has been shown to be involved in many steps that are required for the survival of the parasite inside the insect and establishment of infection in the macrophage (Descoteaux & Turco, 1999; Suvercha *et al*., 2010). The generation of cell mediated immunity is dependent on interaction between APC, DCs and T cells. Berberich *et al*., (2003) have shown that plasma membranes of resting DCs only display few MHC class I and MHC class II molecules and no, or very few, co-stimulatory molecules compared with mature activated antigen-containing DCs, where they display high levels of MHC class I, class II and co-stimulatory molecules which are associated with potent activation of T cell immunity. MHC class I and class II in addition to co-stimulatory molecules remained unchanged in DCs infected with *Leishmania* (Brodskyn *et al*., 2001; Jimenez *et al*., 2010). The regulation of the expression of surface MHC I & II and co-stimulatory molecules on DC infected by *Leishmania* may be dependant on the species of *Leishmania*.

In some species, *Leishmania* parasites enhance CD40-L induced IL-12 production and the expression of co-stimulatory molecules in infected DC. In contrast other species inhibit IL-12 production and down-regulate co-stimulatory molecules (Ghosh & Bandyopadhyay, 2004). Like many other intracellular parasites, *Leishmania* have

adapted several survival mechanisms to overcome effective immune responses via, as an example, the inhibition of pro-inflammatory cytokines essential for T-lymphocyte activation. Infection of macrophages with *Leishmania* parasites has been shown to diminish their microbicidal activities through the production of various immunosuppressive signalling molecules, such as arachidonic acid metabolites, IL-10 and TGF-β (Olivier *et al*., 2005). *Leishmania* infection also interferes with MHC class II antigen and co-stimulatory molecule expression and the antigen presentation ability of host cells. Infection of macrophages with *L. donovani* failed to trigger the expression of CD80 and inhibited antigen presentation (Kaye *et al*., 1995). Also, macrophages infection with *L. donovani* inhibited antigen presentation and reduced their ability to activate T cell responses but did not affect MHC class II antigen expression (Meier *et al*., 2003). In another study, infection of Balb/c macrophage with *L. donovani* reduced activated levels of MHC class II antigen. Collectively, infection with *Leishmania* did not produce a consistent pattern of effects on the expression of MHC class II and co-stimulatory molecules which may be influenced by the species and the model systems used (Reiner, 1987). Martin, *et al*., (2010) have shown that BMDCs from Balb/c mice can express different levels of CD40 with a subsequent effect on regulatory T cell generation where low levels of CD40 expression were required for efficient regulatory T cells generation. DCs expressing low levels of CD40 induced Tregs, whereas DCs expressing high levels of CD40 induced effector T cells, possibly CD8+CD40+ T cells with a contraregulatory activity. The adaptive transfer of the former DC exacerbated whereas the latter significantly reduced *L. donovani* infection in Balb/c mice. Similarly, priming of mice with *Leishmania* Ag-pulsed DCs expressing high levels of CD40 induced host protection against *L.*

donovani challenge. In contrast, priming with the low CD40-expressing DC resulted in aggravated infection as compared with the control mice.

DCs have a critical role in the immunity against *Leishmania* but how they interact with the parasite during infection or vaccination is not fully understood. Activation of DCs can lead to IL-12p70 production which regulates Th1 responses, IFN-γ production and ultimately activates macrophage microbicidal activities. *Leishmania* is capable of inhibiting the migration of DCs *in vitro* as well as in lymphoid tissue, thus inhibiting the presentation of antigen to T-cells (Ato *et al*., 2006; Jebbari *et al*., 2002). DCs are able to phagocytose *Leishmania* parasites irrespective of their developmental phase (metacyclic promastigotes or amastigotes) or whether they are opsonised with either complement C3 component or antibodies. DCs become mature and up-regulate the expression of CD24, CD40, CD54, CD80, CD86, OX40L and MHC class II molecules only when infected with antibody-opsonized promastigotes or amastigotes, suggesting that DCs maturation, at least in this model, was dependent on the status of the ingested parasite (Prina *et al*., 2004). As in the case of macrophages, infection of dendritic cells with *Leishmania* is species specific and affects their antigen presentation efficacy to T cells which ultimately either control or exacerbate the infection. Some species of *Leishmania* enhance, but others down regulate the surface expression of co-stimulatory molecules and cytokine production such as IL-12 (Ghosh & Bandyopadhyay, 2004). Infection of BMDCs with *L. major* but not *L mexicana* amastigotes up regulate the expression of CD80, CD54, and MHC Class II antigen related to DC activation (Bennett *et al*., 2001). Schnitzer *et al*., (2010) have shown that DCs were capable of protection against *L. major*. Briefly, *L. major* antigen loaded DCs that had been exposed to UV irradiation or fixed with paraformaldehyde were to serve as successful vaccine. Also, they have shown the

ability of DC derived exosomes to induce protective immunity against cutaneous Leishmaniasis. The method of antigen presentation to receiver T cells involves uptake of intravenously injected DC fragments into late endosomal compartments of spleen DC in the receiver. *In vitro* studies showed that DC fragments induce T-cell proliferation and IL-12 secretion by splenocytes. The development of a cell free vaccine for immunoprophylaxis against Leishmaniasis is possible. Also, Terrazas, *et al*., (2010) have shown that interfering with the activity of DCs can be one of the most effective ways to induce a safer environment to parasite development.

The critical role of DCs in immunity against *Leishmania* has not been fully understood, and the impact of infection or exposure to *Leishmania* antigens on immune responses has to be determined. Hence, *in vivo* experiments were performed to assess whether protective immunity could be induced by immunisation with DCs loaded with *Leishmania* antigens, and whether protection correlates with CTL activity. The effect of *Leishmania* infection on DCs *in vitro* seems to vary depending on the *Leishmania* species and the model system. In this study the effect of *L. mexicana* infection on MHC Class I and II and co-stimulatory molecule expression in Balb/c mouse bone marrow DCs was investigated. The effect of infection with live *Leishmania* parasite on susceptibility of DCs as targets for CTLs was also investigated in the presence or absence of fungizone, an anti *Leishmania* agent. *Leishmania* specific CTLs were generated by immunisation of mice with SLA2 and culturing splenocytes *in vitro* with SLA as previously demonstrated (Ali *et al*., 2009), the summarizing of experimental work in this chapter shown in (Fig 5.1).

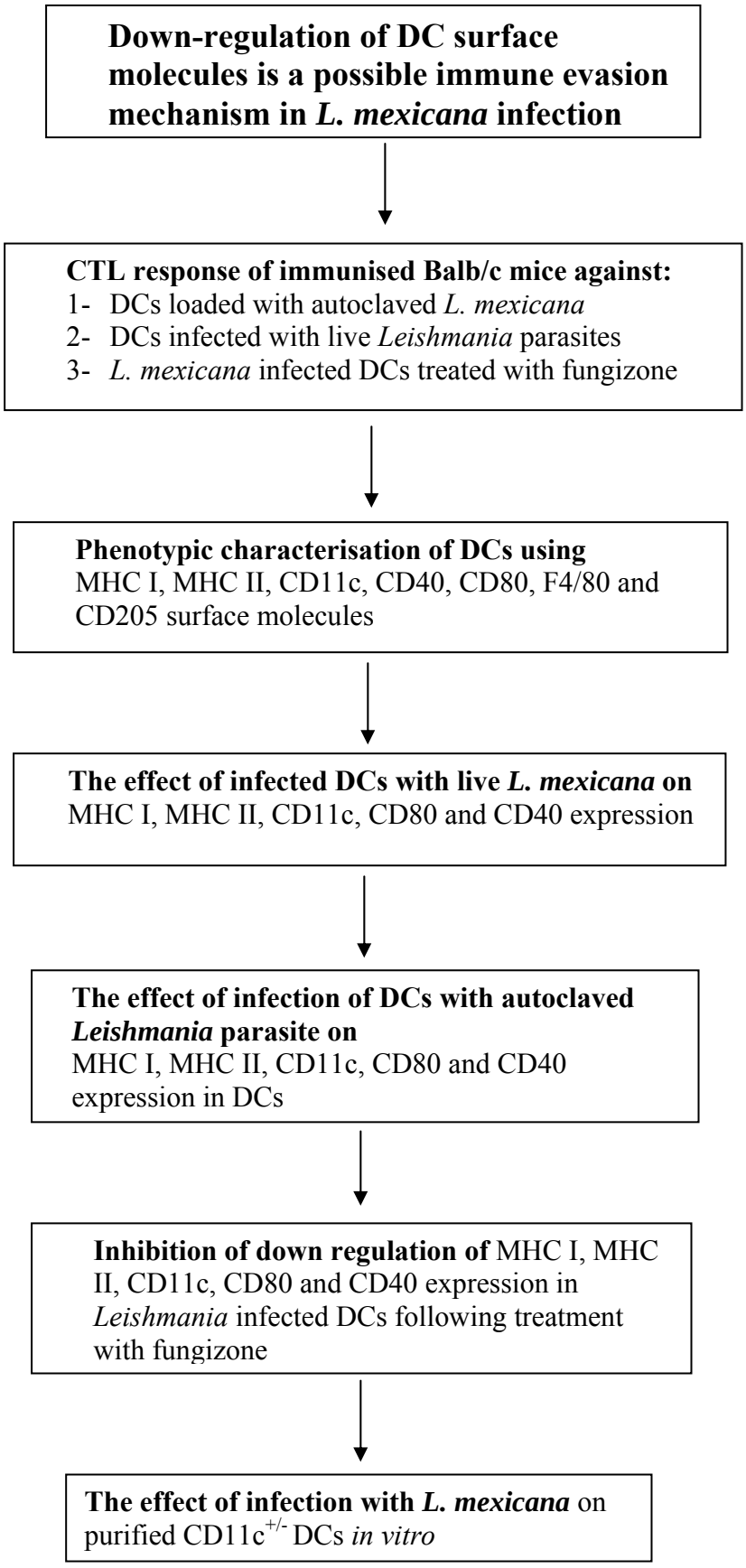


Figure 5.1 A flow chart summarizing the experimental work in this chapter

5.2 Results

5.2.1 CTL activity of immunised Balb/c mice against DCs loaded with autoclaved *L. mexicana* or live *Leishmania* parasites

It has been demonstrated that Balb/c mice immunised with SLA1 mixed with IFA but not DC loaded with SLA1 were significantly protected against challenge with live *L. mexicana* parasite (chapter 3). Surprisingly, *Leishmania* lesions in mice immunised with DCs alone (normal DCs) were delayed in comparison to those given DCs loaded with SLA1. Furthermore, splenocytes from Balb/c mice immunised with *L. mexicana* SLA1 or SLA2 in combination with IFA and mice immunised by DCs loaded with SLA1 or SLA2 induced a potent CTL response compared to controls (chapter 3). In this study CTL activity of Balb/c mice against DCs infected with live parasite in comparison to DCs cultured with autoclaved (killed) parasites was investigated. Balb/c mice were immunised with SLA2 with IFA and in order to generate *Leishmania* specific CTLs, splenocytes were harvested and cultured *in vitro* for 5 days together with naïve cells pulsed with LPS and SLA2 (see chapter 2 methods). On day 5, the splenocytes were used as effectors in a standard 4-hour cytotoxicity assay against mature non-adherent DCs. The mature non-adherent DCs were produced as previously described (chapter 4). DCs were divided into three groups: (A) DC targets cultured with autoclaved *L. mexicana* at a concentration of 10 parasites/DC (B) DC targets infected with live *L. mexicana* at 10 parasites/DC and (C) untreated DC alone as control. The results clearly revealed that DCs loaded with autoclaved *L. mexicana* were susceptible to CTL activity which was positively correlated with MHC class I expression, but DCs loaded with live parasites showed down regulation of MHC I expression and reduced susceptibility to killing (Fig 5.2.1).

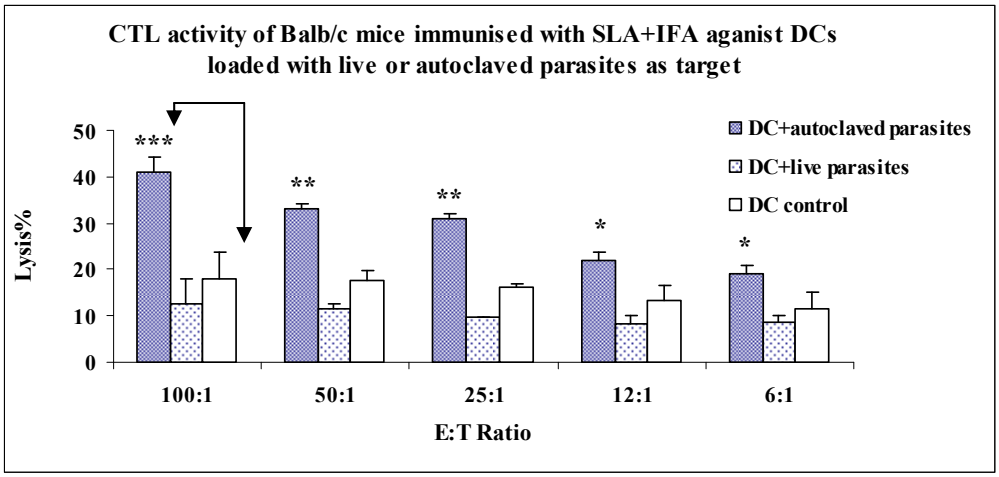


Figure 5.2.1 CTL activity of Balb/c mice immunised with SLA2 against DC target cells incubated with killed *L. mexicana* or live *L. mexicana*
Balb/c mouse was immunised with 100µg/mouse of SLA2. Non-adherent DCs (10 autoclaved or live parasites per DC) were used as target cells. Immunised Balb/c mouse splenocytes were cultured *in vitro* for 5 days together with blast cells pulsed with LPS and SLA2. On day 5 they were used as effector cells in a standard 4-hour cytotoxicity assay. The graph represents 3 independent experiments. Bars represent the standard deviation n=3 p*≤0.05, p**≤0.01 by T test.

5.2.2 Effect of treatment with fungizone of susceptibility of DCs to CTL killing

The susceptibility of DCs infected with live parasites compared to DCs cultured with autoclaved parasites to CTL activity was investigated. Balb/c mice were immunised S.C. with 100µg/mouse SLA2 with IFA. Splenocytes were harvested and cultured *in vitro* for 5 days pulsed with LPS and SLA2. On day 5, the splenocytes were used as effectors in a standard 4-hour cytotoxicity assay against mature non-adherent DCs loaded with SLA2, autoclaved parasites, live parasites and DCs treated 1 hour after infection with fungizone (10 parasites /DC). The results clearly revealed that DCs loaded with autoclaved *L. mexicana* were susceptible to CTL killing which was positively correlated with MHC class I expression (Fig 5.2.2). Treatment with fungizone restored the expression of MHC I and II in the DCs after *Leishmania* infection (Fig 5.2.2).

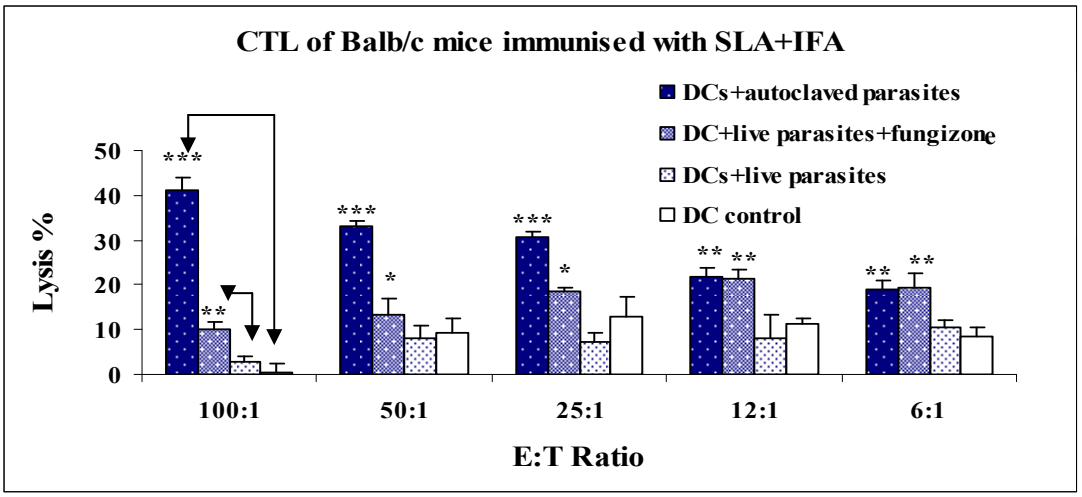


Figure 5.2.2 CTL activity in the presence of fungizone
Balb/c mice were immunised with SLA (100µg/mouse) and Splenocytes were cultured *in vitro* for 5 days together with blast cells pulsed with LPS and SLA. On day 5 they were used as effector cells in a standard 4-hour cytotoxicity assay against DCs + live parasites, DCs infected with live parasites for 1 hour and treated with fungizone overnight, DCs + autoclaved parasites, and non-infected DCs. The graph represents 3 independent experiments. Bars represent the standard deviation n=3 p*≤0.05, p**≤0.01 and p***≤0.001 by T test.

5.2.3 The expression of surface molecules on bone marrow derived cells

The expression of different surface molecules on bone marrow derived cells was investigated. Bone-marrow cells were flushed out of fibula and femur bones with DC media and harvested (see chapter 2 methods). The expression of CD11c, CD40, CD80, F4/80, CD205 MHC I and MHC II on DC cells was determined by staining with corresponding FITC labelled rat anti-mouse antibody and flow cytometry analysis (Table 5.2.3). Different types of bone marrow derived cells: mature adhered, immature adhered, mature non adhered and immature non adhered cells were stained for different cell surface markers, (maturation of DCs was induced by culturing bone marrow cells with LPS overnight and adhered cells were removed by scraping off from the surface of the culture flasks. Results clearly indicate that only mature non adherent cells significantly express all the DC markers such as MHC I, MHC II, CD11c, CD205, CD40 and CD80, and do not express the macrophage marker F4/80. This clearly indicated that out of four different types of cell only mature non adherent

cells contain the highest DC specific markers. According to these findings these cells; mature non adherent bone marrow cells were used as DC BM cells (Fig 5.2.3, Table 2.7).

A:

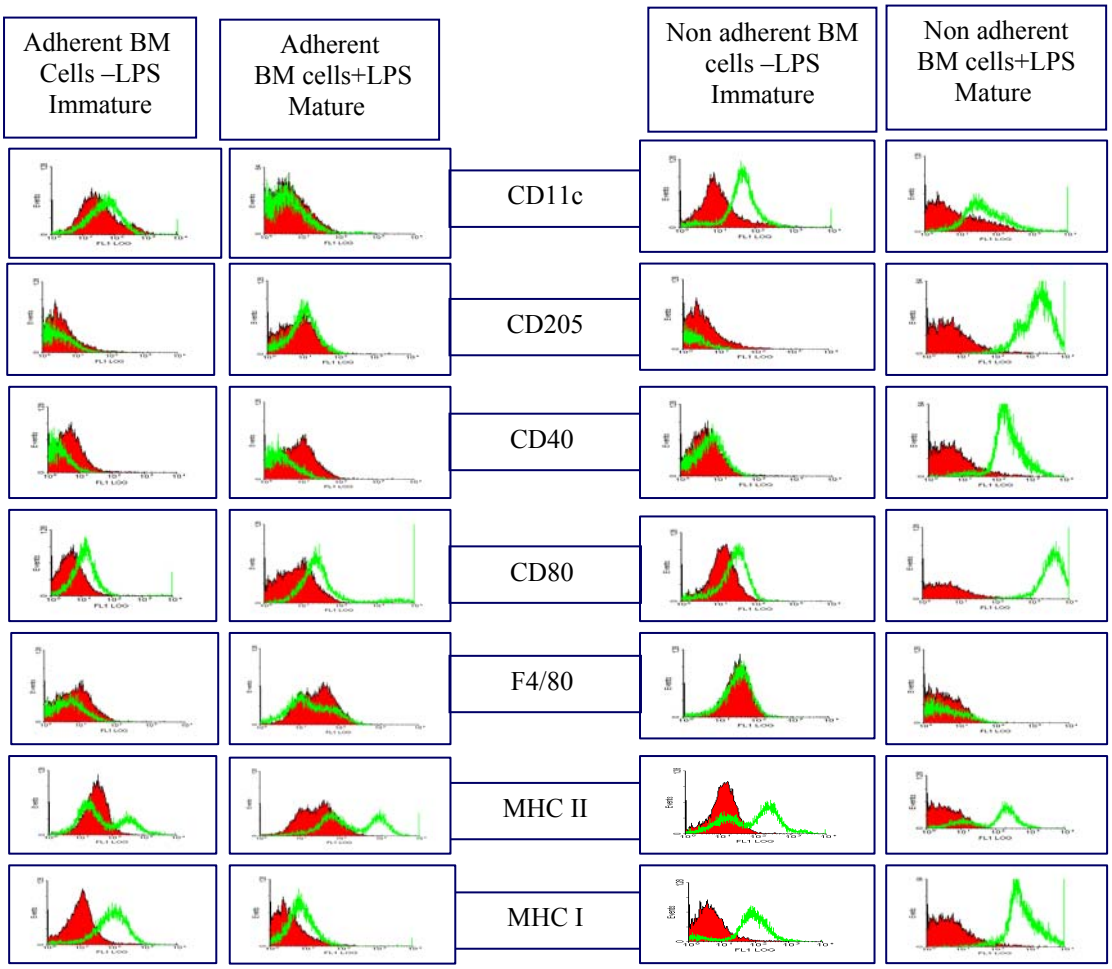


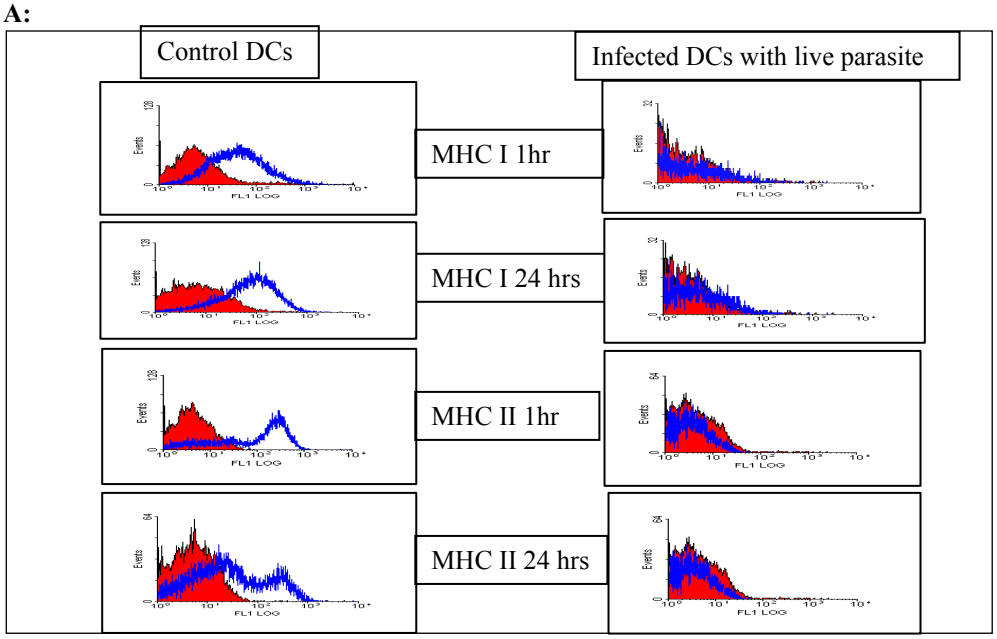
Figure 5.2.3 Phenotyping of the Bone-marrow derived cells subgroups using monoclonal antibodies and flow cytometry analysis
Bone-marrow (BM) cells were cultured in the presence of GM-CSF for 6 days with washes every 2 days. BMs were harvested (non-adherent and adherent cells). Cells were analysed for the expression of MHC class I, MHC class II, CD11c, CD205, F4/80 CD80, and CD40 by flow cytometry using FITC conjugated antibodies red=control, green=test. The graph represents one of 3 independent experiments, (see more results in the appendix figure 1). The graph represents all cells (ungated) in the histograms obtained by the flowcytometer.

5.2.4 Effect of infection of DCs with live *L. mexicana* on MHC class I, MHC class II, CD11c, CD80 and CD40 expression

The effect of *Leishmania* infection on the expression of MHC class I, MHC class II CD11c, CD80 and CD40 was evaluated on bone marrow derived DC (mature non

adherent bone marrow cells) infected with *L. mexicana* (Fig 5.2.4-A,B&C). DCs were cultured as described in section 5.2.3 and were split into two groups. The first group was infected with live *L. mexicana* promastigotes (10:1, parasite: cell) for 1 hour and 24 hours, second group was non infected DCs (control), both groups of DCs were assessed for marker expression by staining with rat anti-mouse CD11c, CD80, CD40, H_2-k^d, H_2-L^d and rat anti-mouse A/E antibody. The results (Fig 5.2.4-A&B) clearly show a down regulation of CD11c, CD80, CD40, MHC class I and class II molecules in *Leishmania* infected DCs compared with controls. It was also shown that the MHC I and MHC II expression were completely down regulated as early as 1 hour after infection. Finally, almost the same level of down regulation at 1 hour and 24 hours was observed.

A:



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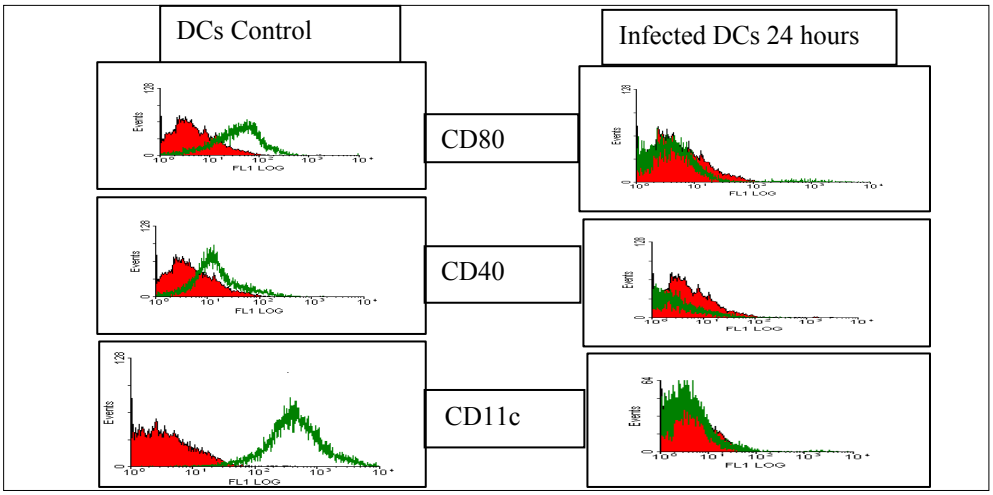
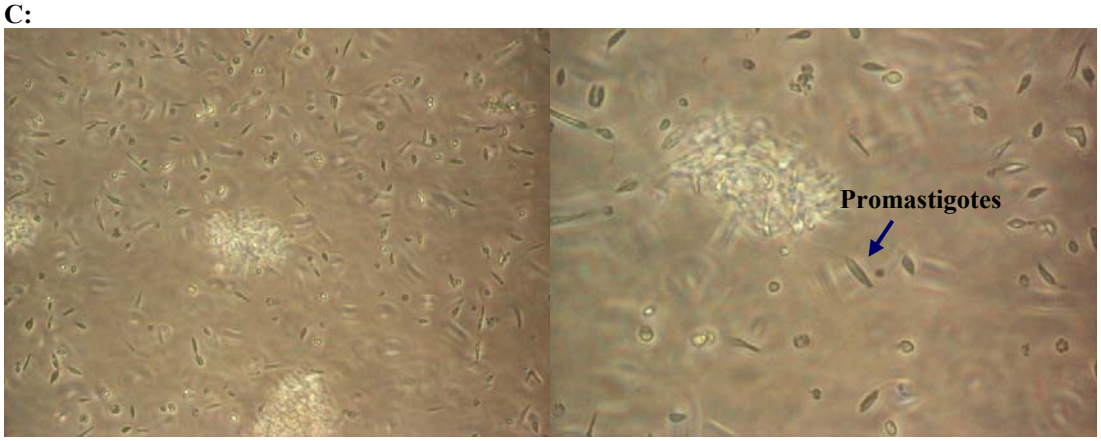


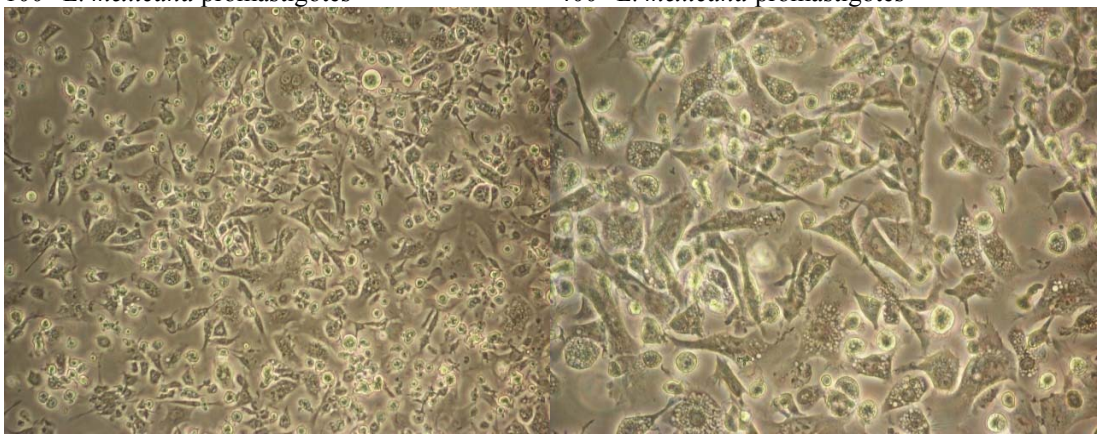
Figure 5.2.4 The effect of *Leishmania* infection on the expression of MHC class I, MHC class II CD11c, CD80 and CD40

A: Expression of MHC class I and class II molecules in *Leishmania* infected DCs: DCs were harvested and split into four groups. Two groups were infected with *L. mexicana* at 10 parasites to one DC for 1 hour or 24 hours. Non infected DCs were used as control. Infected and control DCs were stained for the expression of MHC class I and MHC II using corresponding FITC antibodies. **B: Expression of CD11c, CD80, and CD40 molecules in *Leishmania* infected DCs:** DCs were harvested and split into two groups. The first group was infected with 10 live parasites to one DC for 24 hours. The second group of non infected DCs were used as control. Cells were analysed for the expression of CD11c, CD80, and CD40 by flow cytometry analysis. Red=control, green and blue=test. The graph represents more than one of 3 independent experiments see more result in appendix figure 2).

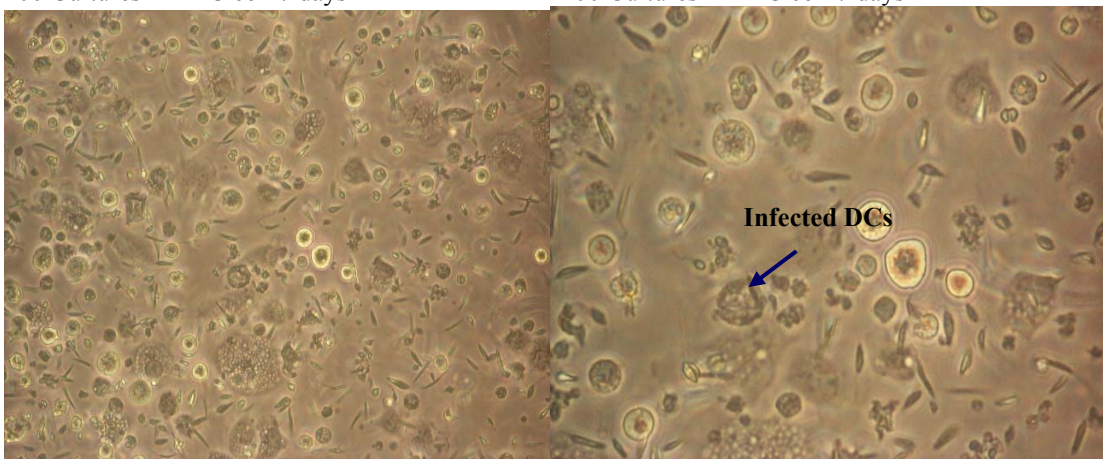
C:



100 *L. mexicana* promastigotes | 400 *L. mexicana* promastigotes



100 Cultures BMDC cell 7 days | 400 Cultures BMDC cell 7 days



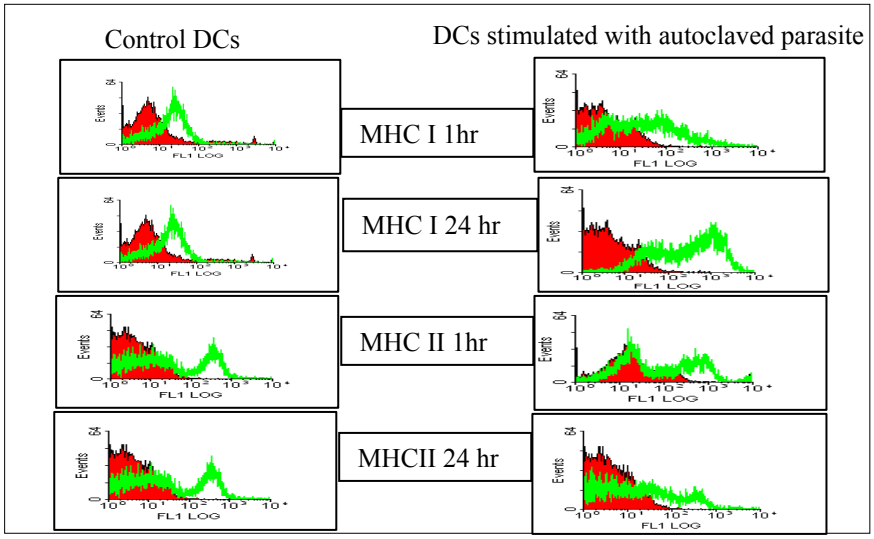
100 Infected DCs with *L. mexicana* | 400 Infected DCs with *L. mexicana*

Figure 5.2.4-C *L. mexicana* promastigotes and DCs infected with *L. mexicana* DCs were harvested and infected with 10 live parasites to one DC. Bone-marrow cells were flushed out with media and harvested. Cells were then centrifuged and resuspended in 1ml BM-DC media, after 7 days DCs were counted and plated at 1×10^6 cells per ml and infected with *L. mexicana* parasites (10 parasite/one DC).

5.2.5 Effect of autoclaved *Leishmania* parasite on the expression of cell surface markers in DCs

Further experiments were conducted to examine whether killed parasites caused the down regulation of surface molecules expressed by DCs. DCs were cultured as described in section 4.2.3 and were split into two groups. The first group was infected at a multiplicity of 10 autoclaved *L. mexicana* promastigotes per DC for 1 hour and 24 hours. DCs were then stained with anti-mouse MHC I, MHC II, CD11c, CD40 and CD80 antibodies and analysed by flow cytometry. The results (Fig 5.2.5-A&B) clearly show that incubation of BM-DCs with killed *L. mexicana* parasites for up to 24 hrs did not inhibit the expression of CD11c, CD40 and CD80 but caused a slight up-regulation of MHC I and MHC II molecules expression. No difference was observed whether BM-DCs were treated with killed parasite for 1 or 24 hours.

A:



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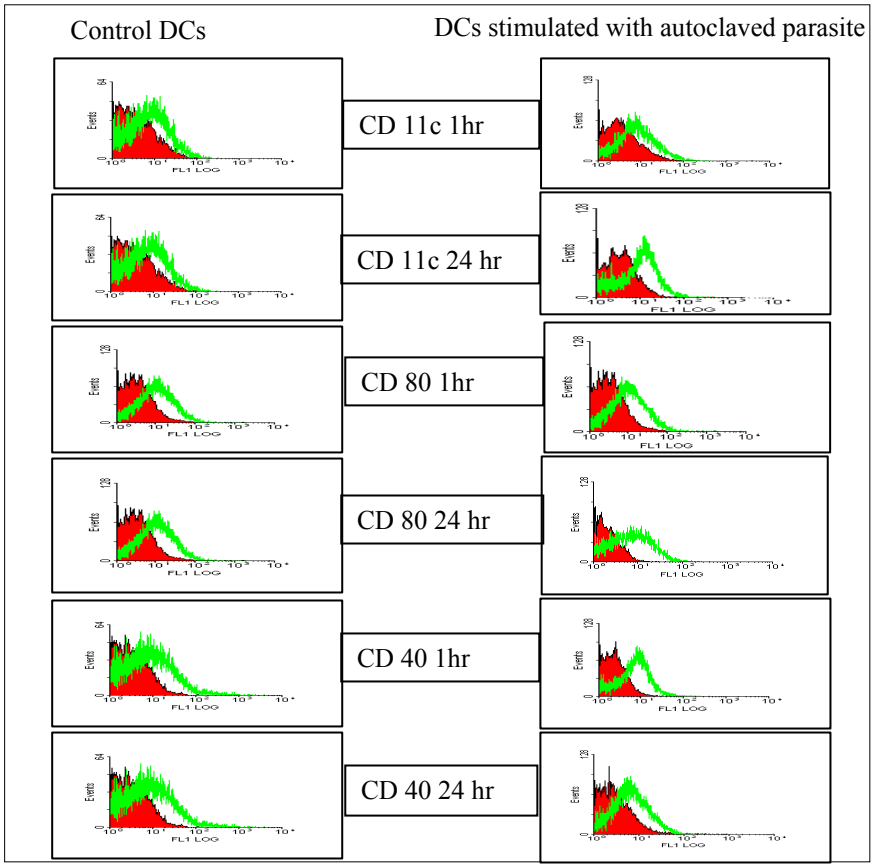


Figure 5.2.5 Effect of autoclaved *Leishmania* parasite on the expression of cell surface markers in DCs

A: Expression of MHC I& II molecules: DCs were harvested and split into two groups. The first group was divided into two groups and stimulated with autoclaved parasites (10:1, parasite: DC) for 1 and 24 hours. The second group of non infected DCs was used as control. Cells were analysed for the expression of MHC class I and II by flow cytometry using FITC conjugated MHC class I and II antibodies and IgG2a and IgG2b as isotype control. **B: Expression of CD11c, CD80, and CD40 molecules**: DCs were harvested and split into two groups and stained for marker expression at 1 and 24 hours. Non infected DCs were used as control. Cells were analysed for the expression of CD11c, CD80, and CD40 by flow cytometry using FITC conjugated antibodies or IgG2a the isotype control. Red=control, green=test. The graph represents one of 3 independent experiments.

5.2.6 The effect of fungizone on the expression of cell surface markers in DCs following *Leishmania* infection.

The infection of DCs by parasites down regulates the expression of MHC class I, class II, CD11c, CD80 and CD40 surface molecules. Here, the effect of fungizone on the expression of MHC class I, MHC class II, CD11c, CD80 and CD40 in the DCs following *Leishmania* infection was also investigated. DCs were cultured as described in section 4.2.3 and split into three groups. The first group was infected with *L. mexicana* promastigotes (10:1, parasite: DC) for 24 hours; the second group was similarly infected but treated after one hour with fungizone at 7.5μg/ml and incubated at 37°C overnight. The third group (non-infected) served as control. On the following day DCs were washed and stained with antibodies for MHC class I, class II, CD11c, CD80 and CD40 and analysed by FACS. The addition of fungizone after one hour infection abrogated the inhibition of MHC class I and class II antigen expression caused by parasitic infection (Fig 5.2.6). Moreover, fungizone restored to normal levels, the expression of CD11c, CD80 and CD40 which restore their susceptibility to CTL activity.

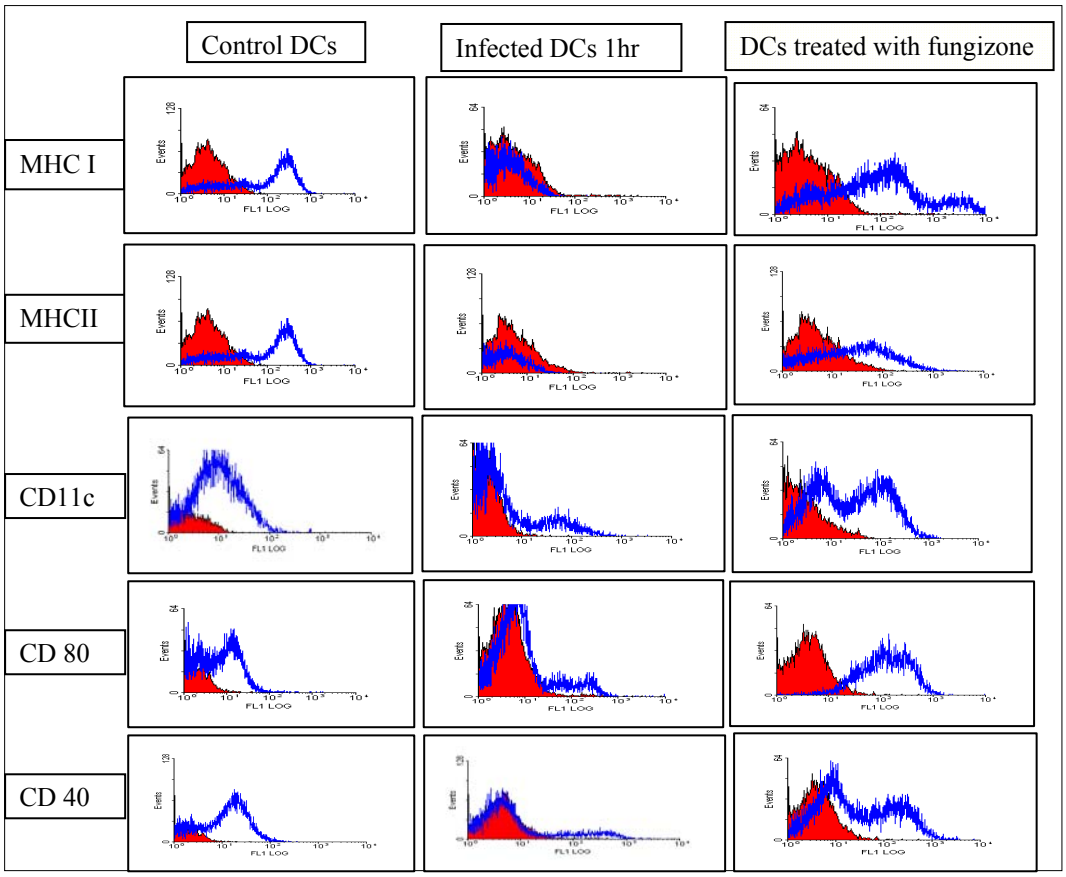


Figure 5.2.6 The effect of fungizone on expression of MHC I, MHC II, CD11c, CD80 and CD40 in DCs following *Leishmania* infection
DCs were harvested and split into three groups. The first group was infected with parasites (10 parasites: 1 DC) for 1 hr and then was treated 24 hrs with fungizone, the second group was infected 24 hrs with parasites (10 parasites: 1 DC). No parasites were added to the third group. Cells were analysed for the expression of MHC I, MHC II, CD11c, CD80, and CD40 by flow cytometry using FITC conjugated antibodies and IgG2a as control for (MHC I CD80, and CD40), IgG2b for (MHC II) or IgG for (CD11c) red=control, blue=test. The graph represents one of 3 independent experiments see more result in appendix figure 4).

5.2.7 Effect of live *L. mexicana* on CD11c+ and CD11c- DCs *in vitro*

In a further experiment, non adherent BM cells were further purified by passing them through a CD11c MicroBeads column to isolate CD11c+ cells. DCs were cultured as described in section 4.2.3. The results in (Fig 5.2.7-A) clearly show that, BM-DCs purified by passing though MicroBeads CD11c column express high level of $CD11c^{+}$ cells compared with controls and infection of purified DCs with live parasites completely down regulate the expression of the cell surface $CD11c^{+}$ marker.

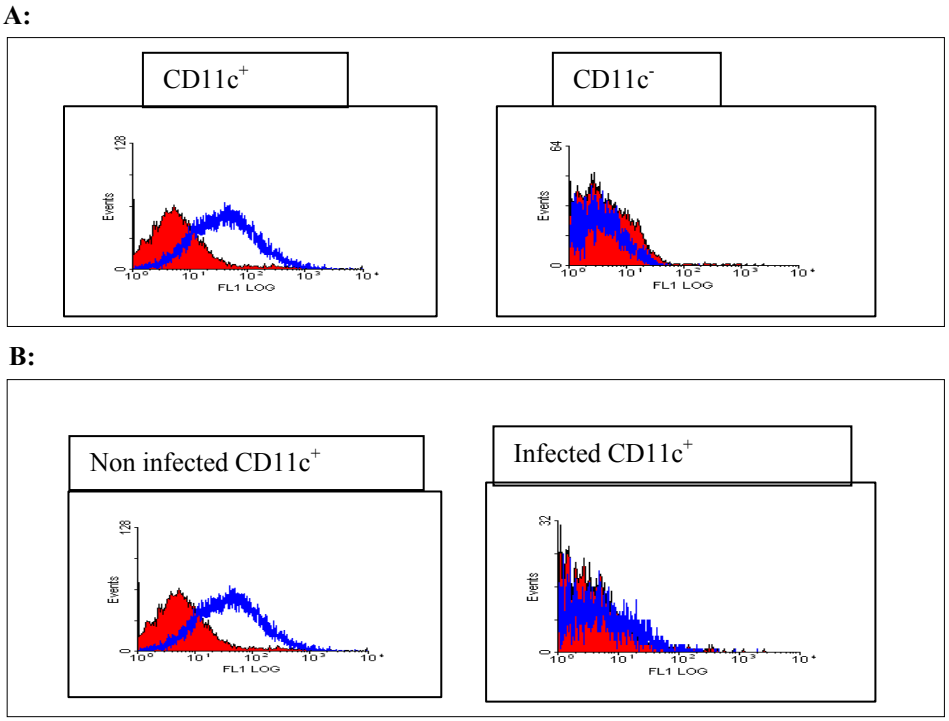


Figure 5.2.7 Effect of live *L. mexicana* on CD11c+ and CD11c- of DCs *in vitro*
A: Analysis of cells following separation: DCs were harvested and separated by CD11c MicroBeads into $CD11c^+$ and $CD11c^-$cells and analysed by flow cytometry using FITC of CD11c antibody. **B: CD11c expression following parasite infection.** Cells were analysed by flow cytometry for the expression of CD11c before and after infection with *L. mexicana*. The graph represents one of 3 independent experiments, red=control, blue=test.

5.3 Discussion

There are many immune evasion mechanisms that assist intracellular parasites to survive inside dendritic cells, macrophages and other host cells. The immune evasion mechanisms adapted by the parasite could diminish host protection against intracellular infection and through active suppression of the synthesis of anti-microbial effector molecules such as cytokines and chemokines (Sacks & Sher, 2002; Ali *et al*., 2009).

This study focused on the effect of *L. mexicana* infection and autoclaved parasite on the expression of MHC I, MHC II, CD40, CD80 and CD11c on the surface of DCs as determined by flow cytometry analysis. Different types of bone marrow derived cells i.e. mature adhered, immature adhered, mature non adhered and immature non adhered cells were stained with antibodies to look for different cell surface markers (maturation of DCs was induced by culturing bone marrow cells with LPS overnight). Results presented in this study have repeatedly shown that only mature non adherent cells express all the DC markers, MHC I, MHC II, CD11c, CD205, CD40 and CD80, and do not express the macrophage marker F4/80. This clearly indicated that out of four different types of cells mature non adherent cells contain the highest level of DC specific markers. Therefore only this set of cells was used for further analysis. Maturation of DCs is crucial for the initiation of immunity since in most tissues where DCs exist in an immature form they are unable to stimulate T cells due to lack of the required accessory signals (Banchereau & Steinman, 1998; Min *et al*., 2010). Results presented in this study demonstrated down regulation of MHC I, MHC II, CD40, CD80 and CD11c at the cell surface of DCs due to *L. mexicana* infection. It

has been previously shown that infection with *L. donovani* induced suppression of MHC class II expression and down-regulation of CD80 as a result of the degradation of MHC class II molecules of the host cell by amastigotes inside the vacuole (parasitophorous) which is exhibiting phagolysosomal properties. However, infection with *L. amazonensis* induced inhibition of antigen processing and peptide loading of MHC molecules. *L. donovani* and *L. major* were also shown to prevent the transport of the MHC-peptide complexes to the cell surface (Reiner 1987; Muraille *et al*., 2010).

The direction of immune response to *Leishmania* (Th1 or Th2) is controlled by the nature and type of *Leishmania* antigen presented and recognised by T cells; therefore, Th1 responses may be initiated by different antigens to those that induce Th2 responses (Awasthi *et al*., 2004). However, various animal studies have implicated that the same parasite epitope may induce a Th1 response in animals with resolving infection and a Th2 response in those susceptible to the disease (Piscopo & Mallia, 2006). Results presented in this study demonstrated that splenocytes from Balb/c mice immunised with SLA2 specifically killed DC targets loaded with autoclaved parasite but not DCs infected with live *L. mexicana* where susceptibility to CTL killing was positively correlated with MHC class I expression. This has suggested that infection with *Leishmania* hinders the target recognition phase of effecter CD8+ T cells. *In vitro* depletion of CD8+ T cells by anti-CD8 Ab significantly inhibited CTL activity though no strong correlation between CTL activity and resistance to infection was found in this model (Ali *et al*., 2009).

It was important to determine whether the down regulation of DC surface molecules, including crucial CTL target ‘MHC’ induced by *L. mexicana* infection, was reversible

and susceptibility of infected DCs could be restored when the infection is eliminated by chemotherapy. Hence fungizone, a non DC toxic anti *Leishmania* agent, was used to effectively treat *L. mexicana* infected DCs in culture. *In vitro* treatment of *L. mexicana* infected DCs with fungizone restored their MHC class I expression as determined by antibody staining and flow cytometry analysis. Interestingly, treatment of *L. mexicana* infected DCs with fungizone also restored their susceptibility to CTL activity. Some studies revealed that infection with *Leishmania* causes the down regulation of the MHC II, which represents a potential immune evasion mechanism adapted by the *Leishmania* (Brandonisio *et al*., 2004). Amprey *et al*., (2004) has shown besides suppression of IL-12, NO, superoxide and the degradation of STAT proteins (Signal Transducer and Activator of Transcription; this protein is a member of the STAT protein family. In response to cytokines and growth factors) *Leishmania* can also regulate various other immune molecules which activate Th1 cells. Also Soong, (2008) has shown that down regulation of IL-12 by *L. mexicana in vitro* could be possibly due to inadequate antigen presentation as a result of MHC down regulation

Soong, (2008) has also reported that the interaction of *Leishmania* parasites with dendritic cells is complex and involves reactions which may induce or inhibit T cell responses, ultimately causing either control or progression of the disease. *Leishmania* infection leads to the obstruction of the up-regulation of the MHC I and II at the transcriptional level. It also down regulates MHC class II antigen by affecting its post translational mechanism; these studies suggest that intracellular amastigotes degrade MHC class II molecules (Bogdan *et al*., 1996; Phillips *et al*., 2010). In contrast, skin DCs from *L. major* infected C57BL/6 or Balb/c mice both show up-regulation of

surface MHC class I and II antigens, CD40, CD54, CD86 and release IL-12p70, thus suggesting that genetic susceptibility to *L. major* is not dependent on DC inability to respond to the parasite (von Stebut *et al*., 2000). However, an increased expression of IL-4R was observed on LCs infected with *L. major* from susceptible but not resistant mice (Moll *et al*., 2002), and the expression of the co-stimulatory molecule CD80 was down-regulated in susceptible but not resistant mice (Mbow *et al*., 2001). Moreover, in lymph nodes, DCs from susceptible, but not resistant mice *in vivo* infected with *L. major*, showed a decreased CD40 activity, which correlated with underproduction of IL-12p40 and IL-12p40 mRNA expression (Heinzel *et al*., 1998; Brandonisio *et al.*, 2004).

Flow cytometry analysis demonstrated up regulation of MHC I, MHC II, CD40, CD80 and CD11c after stimulation of DCs for 1 and 24 hours with autoclaved (killed) parasites, possibly due to some of the components of the autoclaved parasites acting as an adjuvant or immunogen. Furthermore, flow cytometry analysis showed that fungizone treatment of DCs infected with parasites abrogated the suppression of MHC I, MHC II, CD40, CD80 and CD11c on the DCs restoring expression to normal levels. Combination therapy was shown to be effective against many infectious agents, Kleanthous *et al*. 1998 and Jha 2006 demonstrated that the combination of vaccine and partially effective antibiotic therapy was more effective against *H. pylori* infection in an animal model. This is a good example of a successful combination therapy in a microbial model. Leishmaniasis responded much better to treatment with crude vaccine preparations or GM-CSF and IFN-γ mixed with chemotherapy (Raman, 2010). This could be due to enhanced specific immune response by using TLR synergy. Anti-*Leishmania* immune responses in the presence or absence of active disease was assessed in mice following administration of L110f, a well established

Leishmania poly-protein vaccine candidate, in combination with CpG, monophosphoryl lipid A, a TLR4 agonist, or a TLR9 agonist, or a combination of these. Mice treated with L110f plus monophosphoryl lipid A-CpG produced the highest T cell response and reduced parasite burden compared with mice treated with L110f alone or with single adjuvant.

Moreover, (as shown in Fig 5.2.7-A) BMDCs were further purified by passing though MicroBeads CD11c column to isolate $CD11c^{+}$ from DC cells and showed a high levels of $CD11c^{+}$ cells compared with controls. Infection of purified DC cells with live parasites completely down regulated the expression of cell surface CD11c marker expression. It has been shown that in C57BL/6 mice lymph node DCs expressing the DC specific marker CD11c but not $CD11c^{-}$ were usually heavily infected with *Leishmania* parasites. In contrast, some of the $CD11c^{+-}$ infected cells from Balb/c mice were becoming multi-nucleated giant cells with a dramatic accumulation of parasites without expressing differentiation markers at their surface (Misslitz *et al*., 2004). $CD11c^{+}$ DCs from the lymph nodes of *L. major* infected mice express low MHC class II levels and no detectable CD86 expression, which suggests that they might constitute a reservoir of parasites (Muraille *et al*., 2003; Naik, 2010).

In this study, only mature non adherent BMDC cells were shown to express all the DC surface markers, MHC I, MHC II, CD11c, CD205, CD40 and CD80 but not the macrophage marker F4/80. DCs loaded with autoclaved but not infected with live *L. mexicana* were susceptible to CTL killing which was positively correlated to MHC II & I expression, since infection with live *L. mexicana* down regulated the expression of MHC class I expression. Furthermore, treatment with fungizone restored the expression of MHC I and II in the DCs after *Leishmania* infection and made them susceptible to CTL killing.

Chapter 6 Result

Immunogenicity of *L. donovani Centrin-3*

6.1 Introduction

Immunisation with plasmid DNA encoding *Leishmania* antigens represents a promising approach to vaccination against Leishmaniasis as it has intrinsic adjuvant properties, induces both humoral and cell mediated immune responses and results in long lasting immunity (Rodriguez-Cortes *et al*., 2007). Therefore, in light of its many advantages over other vaccination strategies for Leishmaniasis, DNA vaccination could potentially treat and prevent Leishmaniasis. Many vaccine strategies have been pursued, including the use of whole cell lysate, killed, virulent or irradiated parasite (Selvapandiyan *et al*., 2006). *Leishmania* DNA vaccines and purified or recombinant parasite antigens have also been tested. Most of the studied antigens have so far shown a limited degree of effectiveness as a potential vaccine in animal models but little or no protection in humans. New antigen discovery strategies are essential to identify new antigens with potential as a novel vaccine candidate. The immunogenicity of centrin genes, newly identified *Leishmania* antigens, have not been previously studied and very little is known of their biology in *Leishmania*. A DNA-encoding N-terminal domain of the proteophosphoglycan (PPG*)* gene which is a surface-bound protein in both promastigotes and amastigotes was investigated as a vaccine in hamsters against challenge with *L. donovani*. A protection efficiency of about 80% was observed in vaccinated hamsters with more than 6 month survival after challenge. The efficacy was supported by a surge in inducible NO synthase, IFN-γ, TNF-α, and IL-12 mRNA levels along with down-regulation of TGF-β, IL-4, and IL-10. The level of *Leishmania* specific IgG2 was also increased which was indicative of an enhanced cellular immune response. It was concluded that the N-

terminal domain of *L. donovani* PPG is a potential DNA vaccine against visceral Leishmaniasis (Samant *et al*., 2009).

Centrins are cytoskeletal, calcium binding proteins that are localized in the microtubule organising centre (MTOC) of eukaryotic cells (Baron *et al*., 1991). There are a number of eukaryotic centrin genes that have been recently identified, including one in *Chlamydomonas* and *Saccharomyces cerevisiae*; four genes identified in mice, and three in humans (Errabolu *et al*., 1994; Salisbury, 1995). The recently completed genome database for two trypanosomatids, i.e., *Trypanosoma brucei*, the causative agent of African sleeping sickness and *Leishmania*, have revealed five centrin genes in this group of organisms (Selvapandiyan *et al*., 2006). The functions of some of the centrins have been identified, for example, one group of centrins, which includes: *C. reinhardtii* centrin, *Paramecium* centrins 2 and 3, mouse centrins 1 and 2 and human centrins 1 and 2, are involved in centrosome and basal body segregation (Koblenz *et al*., 2003; Ruiz *et al*., 2005). The other group containing *Leishmania* centrin-1, yeast centrin, mouse centrin-3 and human centrin-3, plays a role in centrosome and basal body duplication (Gavet *et al*., 2003; Khalfan *et al*., 2000). The N-terminal non-conserved domain of centrins, which is variable in length, is considered to be responsible for the functional diversity of centrins (Salisbury, 1995; Bhattacharya *et al*., 1993). *L. donovani centrin 3* (*Ldcen-3*) has a significantly smaller N-terminal region compared to centrins from other species (Selvapandiyan *et al*., 2001).

The *Ldcen3* gene is 100% homologous in *L. donovani, L. major* and *L. mexicana*. Therefore, the use of a DNA vaccine to stimulate an immune response against this protein could represent a novel approach to immunise humans against more than one species of the parasite. A DNA vaccine encoding *Ldcen3* could offer protection

against both visceral and cutaneous Leishmaniasis because of heterogeneity in DNA sequence to that of human centrin-3 (Fig 6.1).

Knocking out the centrin-3 (*Ldcen-3*) gene reduced the growth rate of both promastigotes and amastigotes, and reduced survival in human macrophages *in vitro* (Selvapandiyan *et al*., 2001; 2009). Other studies showed that dominant negative expression of centrin proteins by parasites could result in reduced survival in macrophages *in vitro* or in reduced virulence in mice *in vivo* (Antoniazi *et al*., 2000).

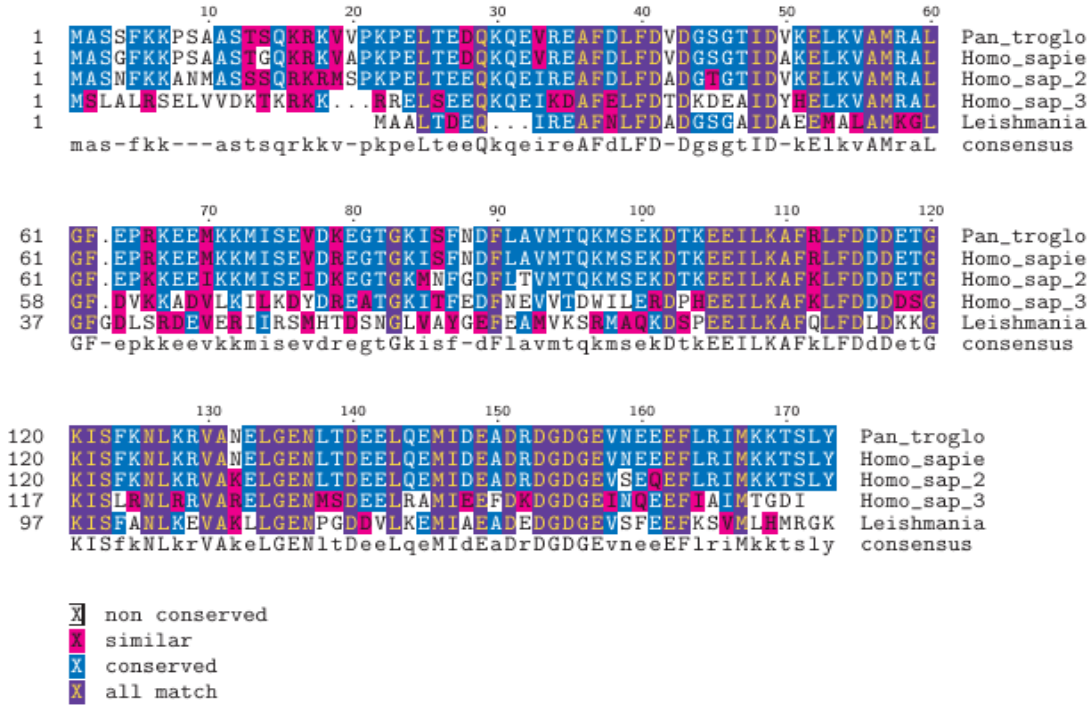


Figure 6.1 The amino acid sequence of *Ldcen-3* compared with human centrins
This figure shows the amino acid sequence of *Ldcen-3* compared with Pan-troglo and human centrin (http:/workbench.sdsc-edu/), Pan-troglo: Chimpanzee.

In the present study the immunogenicity of *Ldcen-3* was investigated in a Balb/c *L. mexicana* model, using a gene gun to release a plasmid DNA construct coated on gold particles. The immunogenicity of *Ldcen-3* has not previously been assessed. The gene gun fires DNA coated gold particles at high velocity directly into epidermal cells,

which consist of skin cells, LC and dermal DC. Inside the cell, plasmid is transported to the nucleus, the encoded gene is transcribed and the protein is subsequently produced, processed into peptides by host proteases and then presented in the context of MHC class I antigen which then stimulates CD8+ T cells (Encke *et al*.,1999; ALi *et al.*, 2009). DC directly transfected with DNA vaccine can prime CD8+ cells by presenting the DNA encoded antigen via MHC class I. Immature DC can endocytose soluble proteins and debris from apoptotic transfected cells and express the coded antigen through MHC class I or MHC class II following differentiation into mature DC. Thus, a DNA vaccine can be effective in the stimulation of both CD8+ T cells and CD4+ T cell populations. The ability of DCs to present extracellular antigens into MHC class I and MHC class II is known as cross priming, accordingly DCs play an important function in the induction of both humoral and cell mediated immunity following DNA vaccination, the summarizing of experimental work in this chapter shown in (Fig 6.1).

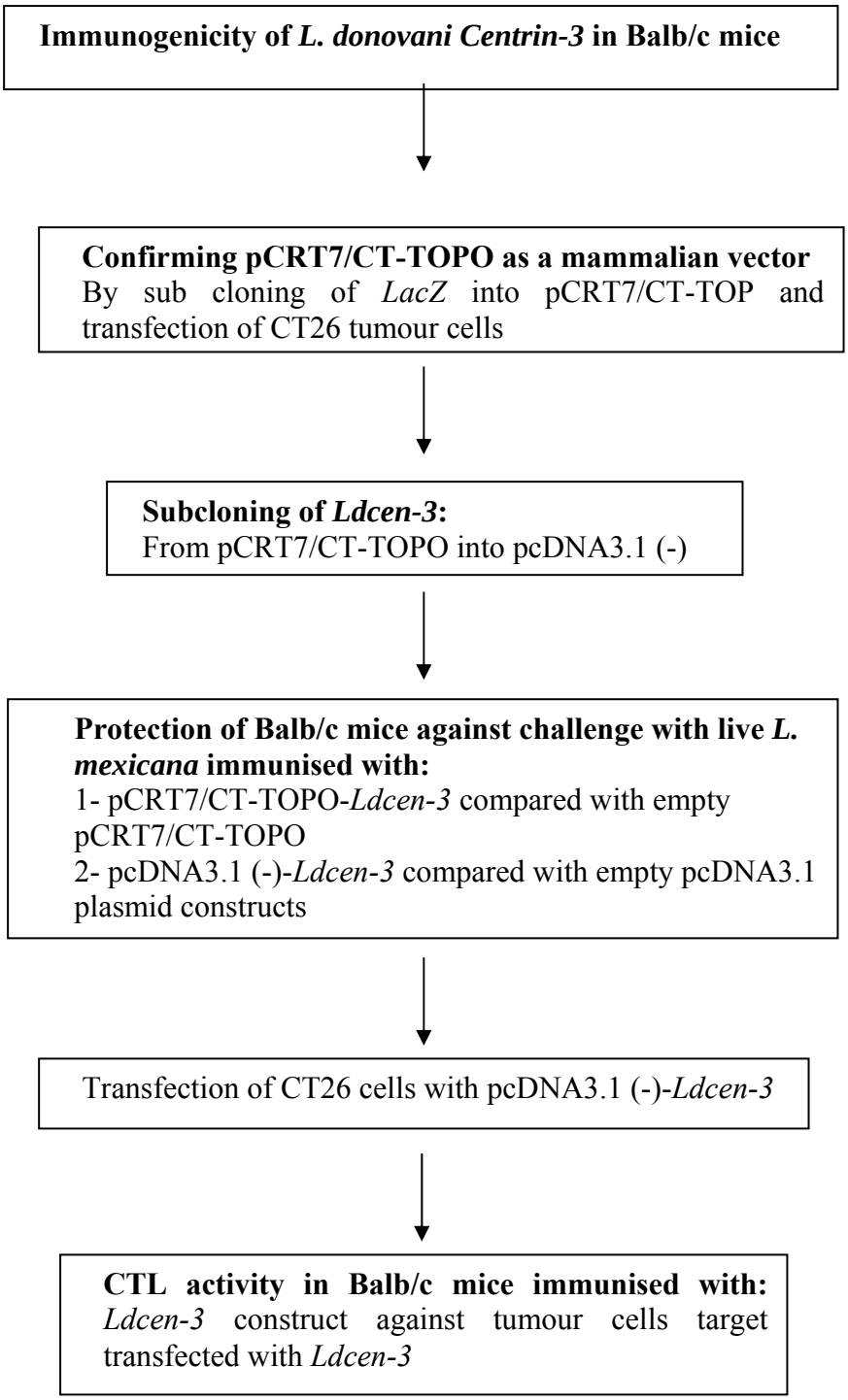


Figure 6.1 A flow chart summarizing the experimental work in this chapter.

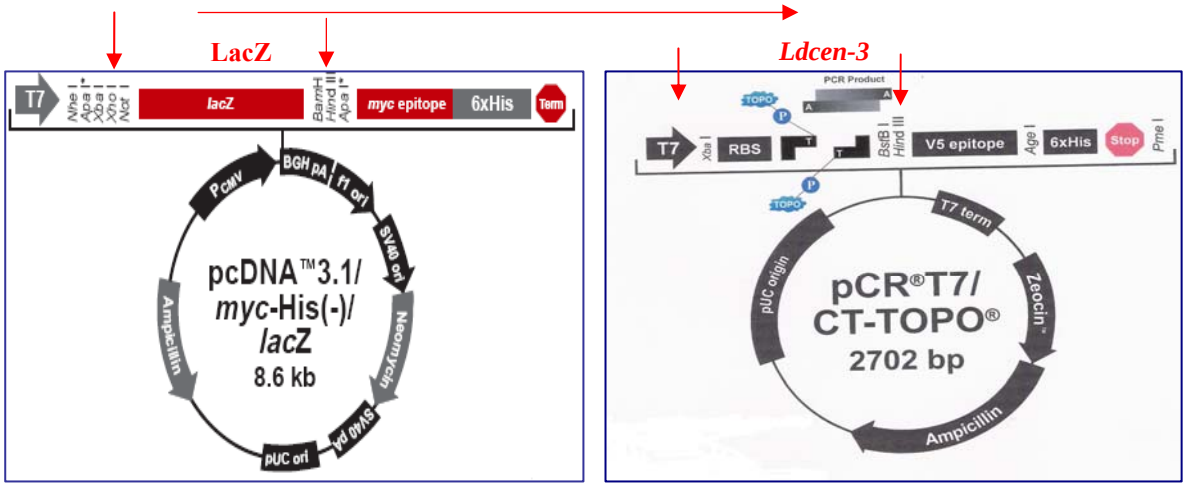
6.2 Results

6.2.1 Confirming pCRT7/CT-TOPO as a mammalian vector

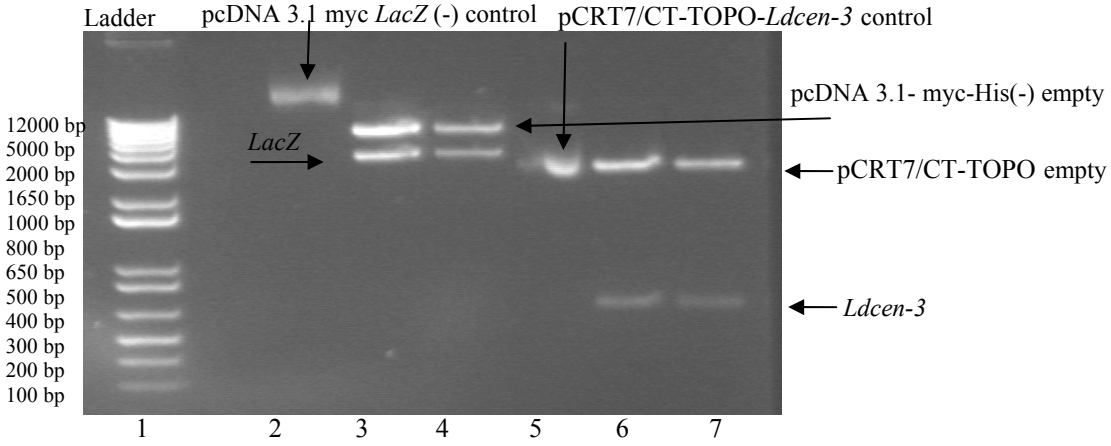
6.2.1.1 Sub cloning of *LacZ* into pCRT7/CT-TOPO

To provide evidence that pCRT7/CT-TOPO-*Ldcen-3* could transfect and express genes of interest in mammalian cells, pCRT7/CT-TOPO-*Ldcen-3* was constructed incorporating the *lacZ* gene as a marker and was used to transfect a mammalian cell line (Fig 6.2.1.1). Briefly, the *lacZ* gene was cut from pcDNA3.1myc-His *lacZ* (-) vector from both sides using *Xba*I and *Hind* III restriction enzymes. The digested fraction was separated by gel electrophoresis (1.5 %). The pCRT7/CT-TOPO vector was also digested using the same restriction enzymes (Fig 6.2.1.1-A, B, C&D). The *Ldcen-3* was extracted from the gel and inserted in pcDNA 3.1(-) as section 6.2.2.2), and then the *lacZ* gene and the empty pCRT7/CT-TOPO vectors were ligated using a DNA ligase enzyme. The pCRT7/CT-TOPO-*lacZ* vector was transfected in CT26 cells to establish the expression ability in mammalian cells; a suitable CT26 clone 25 (CT 26-*lacZ*) mouse tumour cell line stably transfected with *lacZ* gene, was used as a positive control.

A:



B:



C:

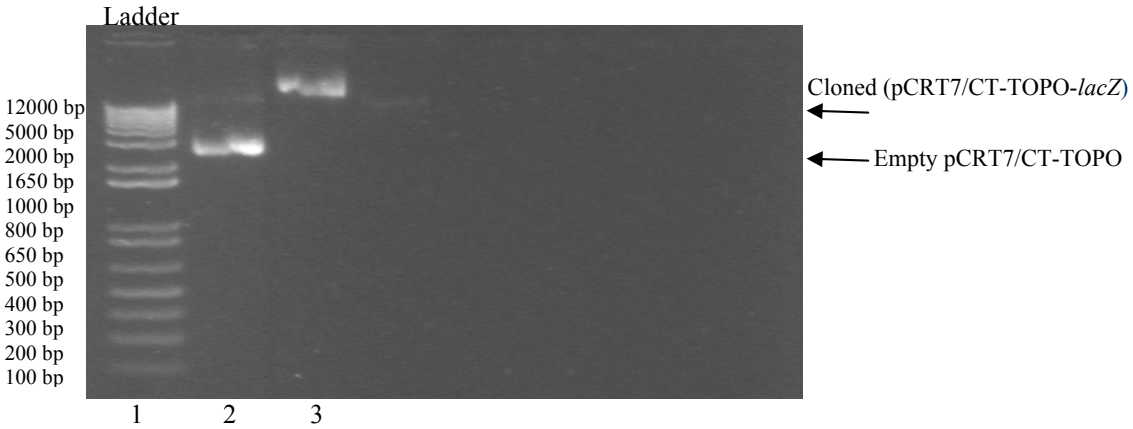


Figure 6.2.1.1 Sub cloning of *lacZ* into pCRT7/CT-TOPO

A: Map representing pCRT7/CT-TOPO and pcDNA 3.1 myc-His/*lacZ*: pCRT7/CT-TOPO vector containing -*Ldcen-3* gene and pcDNA 3.1 myc-His *LacZ* (-) to sub clone *LacZ* in pCRT7/CT-TOPO.**B: Digestion of pcDNA 3.1 and pCRT7/CT-TOPO:** Both plasmids were cut by *Xba*I and *Hind* III **restriction enzymes**: 1- ladder; 2- pcDNA 3.1 myc *lacZ* (-); 3, 4-the above band is the linear – empty pcDNA 3.1 myc-His and lower band is *lacZ*; 5, pCRT7/CT-TOPO-*Ldcen-3* control; 6, 7, above band is the linear empty vector pCRT7/CT-TOPO and lower band is *Ldcen-3*. **C: Sub cloning of *lacZ* into the pCRT7/CT-TOPO vector:** The *lacZ* gene and the digested pCRT7/CT-TOPO vector were collected, purified and ligated using a DNA ligase enzyme, 1- 1kd ladder; 2- empty pCRT7/CT-TOPO and 3- pCRT7/CT-TOPO-*lacZ* (maps and vectors were obtained from invitrogen).

6.2.1.2 Transfection of CT26 cells with pCRT7/CT-TOPO- *lacZ*

β-galactosidase, an important reporter gene encoded by the *lacZ* gene, is commonly used for monitoring transfection efficiency in mammalian cells. The β-galactosidase staining kit was used to determine the expression of *lacZ* following transient or stable transfection of plasmids encoding *lacZ.* β-galactosidase catalyzes the hydrolysis of X-gal producing a blue colour. Transfected CT26 with pCRT7/CT-TOPO-*lacZ* was examined under a light microscope for the development of blue stain, which successfully produced a blue colour when compared to control cells CT26-*lacZ* indicating the ability of pCRT7/CT-TOPO to express *lacZ* in mammalian cells (Fig 6.2.1.2).

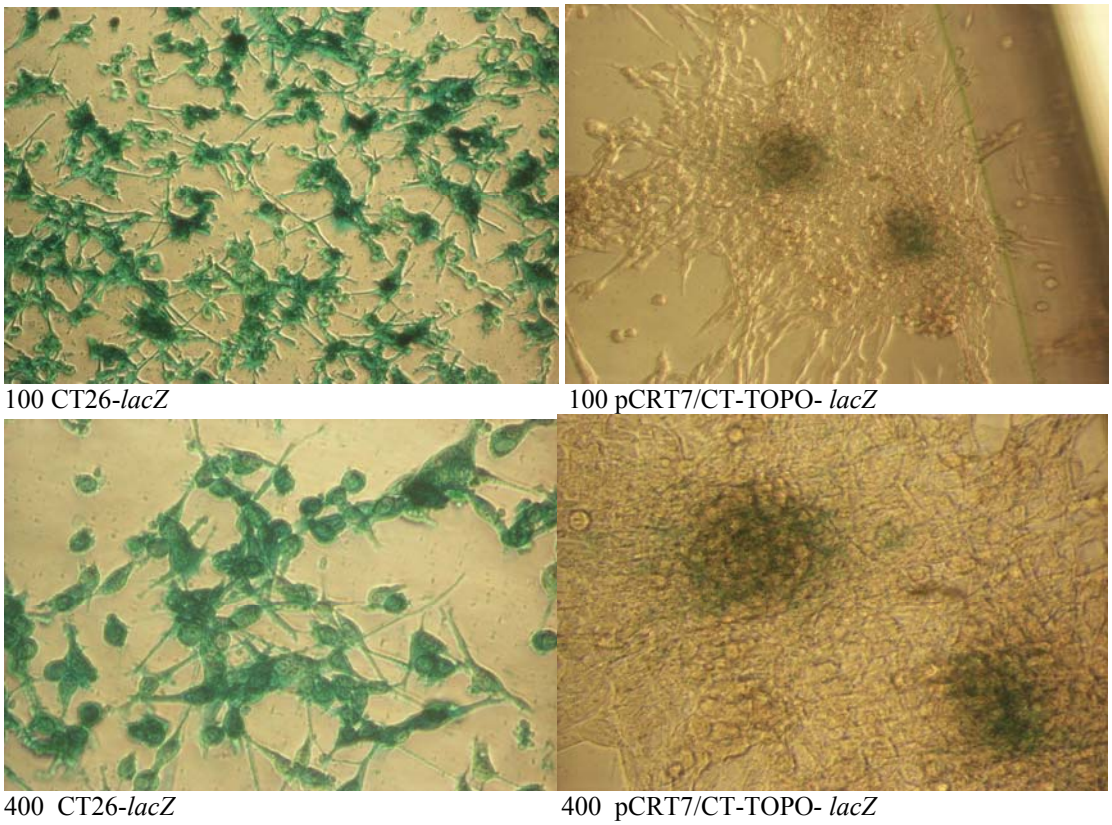


Figure 6.2.1.2 Expression of β-gal in CT26 transfected with pCRT7/CT-TOPO-*LacZ*
CT26 cells transfected with a pCRT7/CT-TOPO-*LacZ* vector were washed twice with PBS and then fixed for 15 minutes with glutaraldehyde. Cells were then stained with X-gal for overnight to test the expression of pCRT7/CT-TOPO in mammalian cells. Blue colour staining indicates the expression of *lacZ*; suitable CT26-clone 25, (a stable transfectant with high expression of B-galactosidase) was used as a positive control.

6.2.2 Subcloning of *Leishmania donovani* centrin-3 (*Ldcen-3*) into pcDNA3.1

6.2.2.1 Confirmation of the presence of *Ldcen-3* by PCR

The pCRT7/CT-TOPO-*Ldcen*-3 vector was bulked up to obtain sufficient quantities of the plasmid and PCR was used to confirm *Ldcen-3* presence using two primers designed for *Ldcen-3* (541bp), *Ldcen-3*F, forward *5`AGA GGC ATT CGT GTT CG-3`* and *Ldcen-3*R, reverse *5`AGG TTG ATC TCG CCA TCT TGA -3`* (Fig 6.2.2.1).

To determine the sequence of the *Ldcen-3* gene, the DNA sample along with two primers that were used for the PCR amplification were sent to MWG-biotech.com for sequencing (Fig 6.2.2.1-B). This confirmed the presence of the pCRT7/CT-TOPO-*Ldcen-3* gene insert.

A:

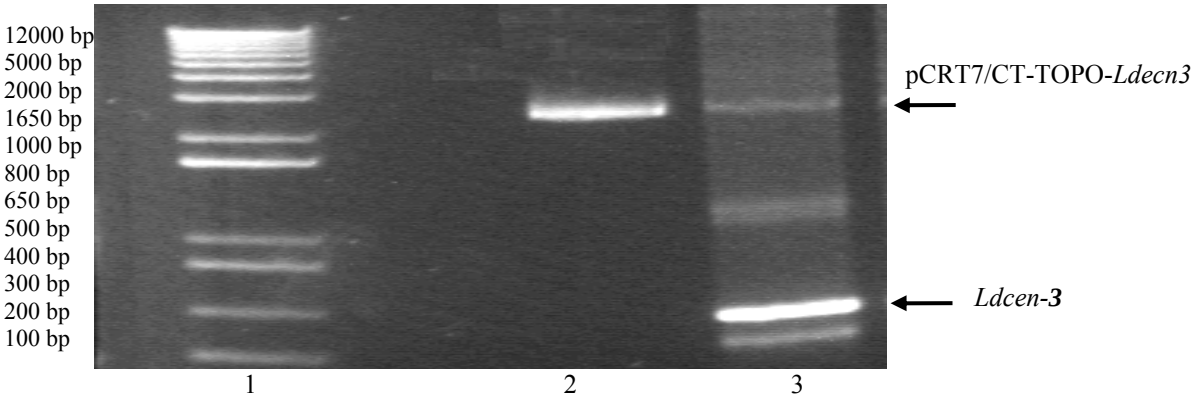


Figure 6.2.2.1 Confirmation of the presence of *Ldcen-3* by PCR
A: Confirmation of the presence of *Ldcen-3* by PCR: The presence of *Ldcen-3* was confirmed by PCR amplification using 5`AGA GGC ATT CGT GTT CG-3` forward and 5`AGG TTG ATC TCG CCA TCT TGA -3`reverse primers 1- ladder, 2- pCRT7/CT-TOPO-*Ldecn3* as a control and not PCR result*, 3- Ldcen-3* **(**PCR product).

6.2.2.2 Subcloning of *Ldcen-3* into pcDNA3.1 (-)

In order to adopt a widely used mammalian vector for DNA immunisation and also to transfect CT26 tumour cells it was decided to sub-clone the *Ldcen-3* gene into a pcDNA3.1 (-) vector (Fig 6.2.2.2), which contained a mammalian selectable marker antibiotic gene. *Ldcen-3* was cut from both sides by *Xba*I and *Hind* III restriction enzymes from the pCRT7/CT-TOPO vector. The digested fractions were separated by gel electrophoresis (Fig 6.2.2.2.1-A). The pcDNA3.1 (-) vector was also cut using the same restriction enzymes. The *Ldcen-3* gene and the digested pcDNA 3.1 (-) vectors were then ligated using a DNA ligase enzyme. The presence of the *Lcen-3* gene in the pcDNA3.1(-) vector was determined by restriction enzyme digestion (Fig 6.2.2.2.1-B) and PCR amplification (Fig 6.2.2.2.1-C) using forward and reverse primers *Ldcen-3*F *5`AGA GGC ATT CGT GTT CG-3`* and *Ldcen-3*R, reverse *5`AGG TTG ATC TCG CCA TCT TGA -3`*.

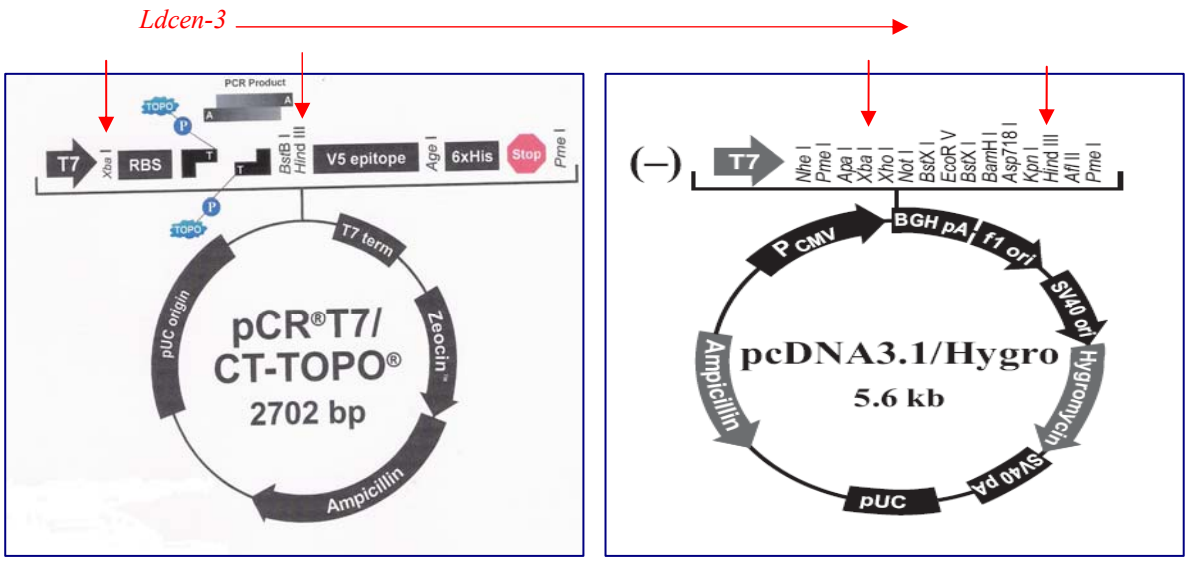


Figure 6.2.2.2 Map representing pCRT7/CT-TOPO-*Ldcen-3* and pcDNA 3.1(-) vectors
pCRT7/CT-TOPO plasmid vector containing *Ldcen-3* gene and pcDNA 3.1(-) for sub cloning of *Ldcen-3*.

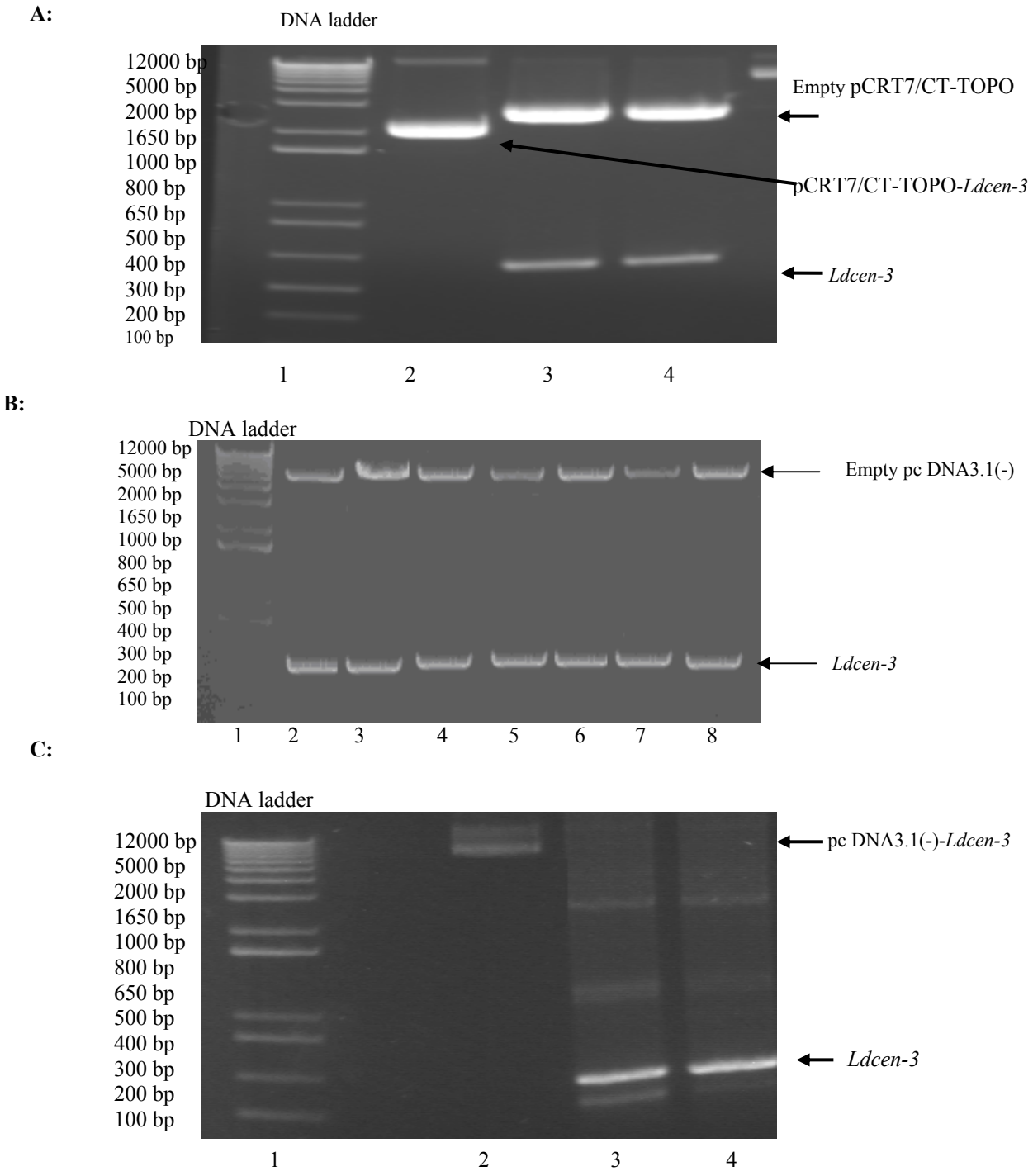


Figure 6.2.2.2.1 Subcloning of *Ldcen-3* into pcDNA3.1 (-)
A: subcloning of *Ldcen-3* into pcDNA 3.1 (-) vectors: pCRT7/CT-TOPO-*Ldcen-3* was cut by *Xba*I and *Hind* III restriction enzyme 1- ladder; 2- pCRT7/CT-TOPO–*Ldcen-3*, 3,4-the above band is a linear empty pCRT7/CT-TOPO and lower band is *Ldcen-3* **B: Confirmation of the presence of *Ldcen-3* in pcDNA3.1 (-):** Restriction digestion with the same enzymes (*Hind* III and *Xba*I restriction enzymes), 1-ladder, 2-8 the upper bands are linear empty pcDNA3.1 (-) and the lower bands are Ldcen-3 after digestion of pcDNA3.1 (-)-*Ldcen-3* with *Hind* III and *Xba*I. **C: Confirmation of the presence of *Ldcen-3* in pcDNA3.1 (-) by PCR**: The presence of *Ldcen*-3 gene was confirmed by PCR using *Ldcen-3* forward primer F 5`AGA GGC ATT CGT GTT CG-3` and the *Ldcen-3* reverse primer R, 5`AGG TTG ATC TCG CCA TCT TGA-3`, 1-DNA ladder, 2-empty pcDNA3.1 (-)-*Ldcen-3* and 3, 4 *Ldcen-3*.

Moreover, to ensure that the sub-cloned gene is complete and no mismatches had occurred during the cloning procedure the whole gene as compared with gene bank (http://www.JustBio.com), (which is shown below) was also confirmed by sequencing (Fig 6.2.2.2.2).

Seq1: the first sequenced gene (published gene bank)

Seq2: new sequenced gene (sub-cloned)

```
sequence1     ATGAACATCACTAGTCGCACATCGGGGCCGCTGCGCACCACTGCGCCGGCGGCATCAGCG
sequence2     ATGAACATCACTAGTCGCACATCGGGGCCGCTGCGCACCACTGCGCCGGCGGCATCAGCG
              ************************************************************************

sequence1     CCGTCCGCGGCAGCGCGCCGTCGCTTCCAGCTTACGGAGGAACAGCGCCAGGAGATCCGA
sequence2     CCGTCCGCGGCAGCGCGCCGTCGCTTCCAGCTTACGGAGGAACAGCGCCAGGAGATCCGA
              ************************************************************************

sequence1     GAGGCATTCGAGCTGTTCGACTCGGATAAGAACGGACTCATCGATGTGCATGAGATGAAG
sequence2     GAGGCATTCGAGCTGTTCGACTCGGATAAGAACGGACTCATCGATGTGCATGAGATGAAG
              ************************************************************************

sequence1     GTCAGCATGCGAGCACTTGGCTTTGATGCAAAACGGGAGGAGGTGCTGCAGCTCATGCAG
sequence2     GTCAGCATGCGAGCACTTGGCTTTGATGCAAAACGGGAGGAGGTGCTGCAGCTCATGCAG
              ************************************************************************

sequence1     GACTGCGCTGCCCGGGACCAGAACAATCAGCCGCTTATGGACTTACCGGGCTTCACAGAT
sequence2     GACTGCGCTGCCCGGGACCAGAACAATCAGCCGCTTATGGACTTACCGGGCTTCACAGAT
              ************************************************************************

sequence1     ATCATGACGGACAAGTTTGCGCAGCGCGATCCTCGGCAGGAGATGGTGAAGGCGTTTCAG
sequence2     ATCATGACGGACAAGTTTGCGCAGCGCGATCCTCGGCAGGAGATGGTGAAGGCGTTTCAG
              ************************************************************************

sequence1     CTGTTTGACGAGAACAATACCGGCAAAATCTCCCTTCGCTCGCTGCGTCGTGTGGCGCGG
sequence2     CTGTTTGACGAGAACAATACCGGCAAAATCTCCCTTCGCTCGCTGCGTCGTGTGGCGCGG
              ************************************************************************

sequence1     GAACTGGGCGAGAACATGAGCGACGAAGAGCTGCAGGCAATGATTGACGAGTTTGACGTA
sequence2     GAACTGGGCGAGAACATGAGCGACGAAGAGCTGCAGGCAATGATTGACGAGTTTGACGTA
              ************************************************************************

sequence1     GATCAAGATGGCGAGATCAACCTAGAAGAGTTTCTTGCCATTATGCTAGAGGAGGACGAC
sequence2     GATCAAGATGGCGAGATCAACCTAGAAGAGTTTCTTGCCATTATGCTAGAGGAGGACGAC
              ************************************************************************

sequence1     TAC
sequence2     TAC
               ***
```

Figure 6.2.2.2.2 Confirmation of the presence of *Ldcen-3* in pcDNA3.1 (-) by sequencing
The sub-cloned gene (*Ldcen-3*) is completed and no mismatches had occurred during the cloning procedure the whole sequencing of *Ldcen-3* gene as compared with gene bank (http://www.JustBio.com).

6.2.3 Construction of pCRT7/CT-TOPO empty vector

In order to produce a pCRT7/CT-TOPO empty vector to be used as a negative control in DNA vaccination and protection studies, the *Ldcen-3* gene was cut and removed from this vector. The *Ldcen-3* gene was cut out from the vector by digestion with *XbaI* and *Hind III* restriction enzyme and the product was run into an agarose gel. The band related to the vector was extracted from the gel (Fig 6.2.3). Both free ends of the vector that resulted from digestion were then ligated to each other by the ligase enzyme. The absence of the *Ldcen-3* gene in the empty vector was confirmed by PCR using *Ldcen-3* primers.

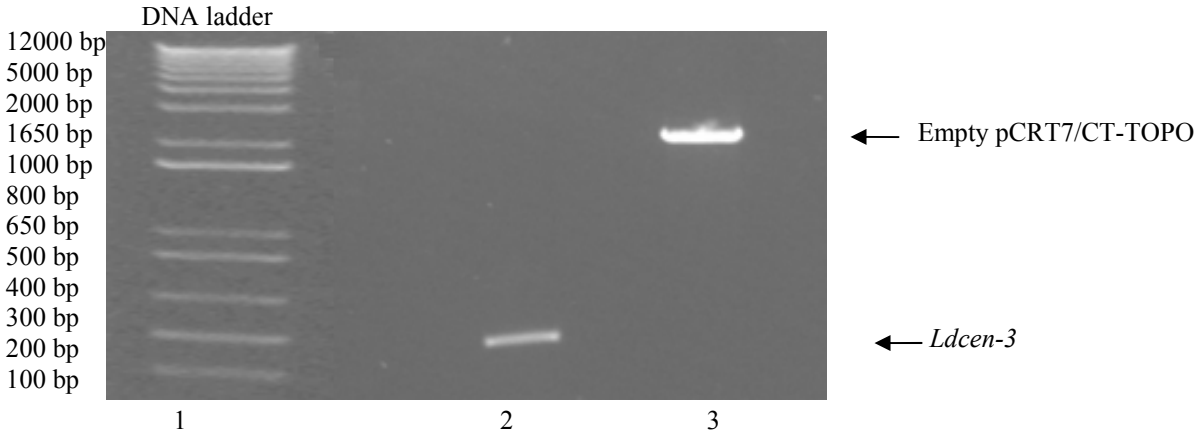


Figure 6.2.3 Production of empty pCRT7/CT-TOPO vector
The pCRT7/CT-TOPO-*Ldcen-3* was digested with *Hind* III and *Xba* I restriction enzymes, and then were extracted *Ldcen-3* and empty vector pCRT7/CT-TOPO after extraction, 1- DNA ladder, 2- emptypCRT7/CT-TOPO and 3- *Ldcen-3*.

6.2.4 Immunogenicity of *Ldcen-3*

6.2.4.1 Protection induced by immunisation with pCRT7/CT-TOPO- *Ldcen-3* plasmid construct

To determine the immunogenicity of *Ldcen-3* (*L. donovani centrin-3*), a pCRT7/CT-TOPO-*Ldcen-3* was used as a DNA vaccine in a Balb/c mouse model (see chapter 2 methods). *L. mexicana* gp63 construct (VR1012-gp63) was used as a positive control

since this gene (*L. mexicana* gp63) was shown to induce strong immunity by DNA-gene gun immunisation (Ali, *et al* 2009). The results (Fig 6.2.4.1-A&B) clearly demonstrated that mice immunised with *Ldcen-3* or gp63 were significantly protected against challenge with live parasites, 5 out of 6 mice were lesion free in *Ldcen-3* or gp63 groups.

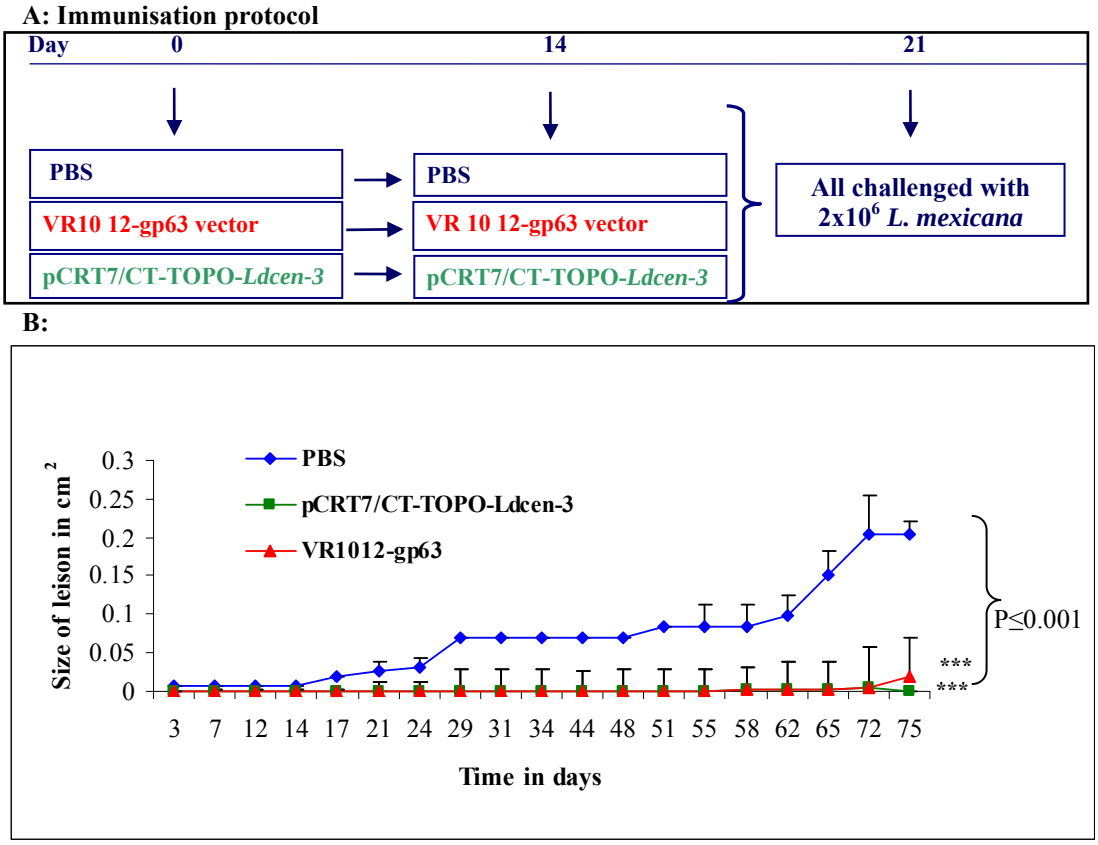


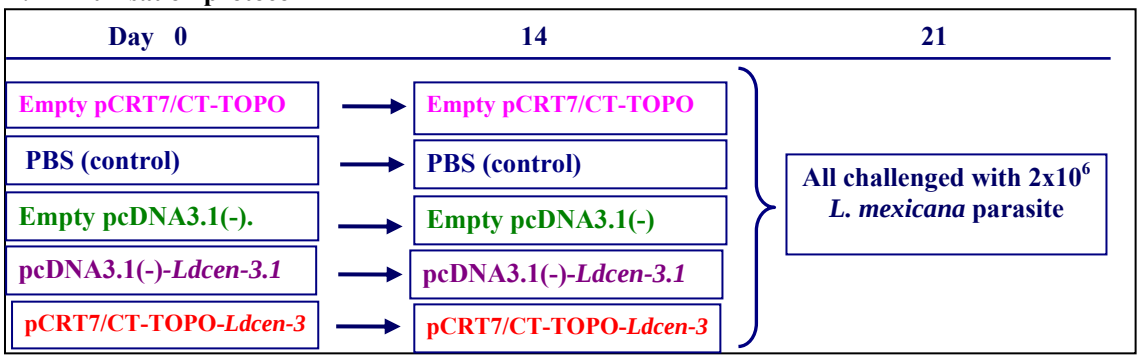
Figure 6.2.4.1 Protection induced by immunisation with pCRT7/CT-TOPO- *Ldcen-3* constructs
A: Immunisation protocol, B: Protection induced by immunisation with pCRT7/CT-TOPO-*Ldcen-3* construct**:** Two groups of Balb/c mice were immunised either with centrin-3 (in pCRT7/CT-TOPO) or gp63 construct by gene gun (1µg/mouse) on days 0 and 14. A third group of Balb/c mice was given PBS and used as control. Seven days after last immunisation mice were challenged with 2×10^6 *L. mexicana* promastigotes. Mice were regularly monitored at least twice a week to determine the lesion size on each mouse. The graph represents 2 independent experiments. Bars represent the standard deviation n=6, P***≤0.001 by T test.

6.2.4.2 Protection induced by immunisation with pCRT7/CT-TOPO- *Ldcen-3* and pcDNA3.1-*Ldcen-3* plasmid construct

The immunogenicity of *Ldcen*-3 cloned in two different vectors was investigated to confirm the immunogenicity to *Ldcen-3*. Balb/c mice were immunised by gene gun

with 1µg of pcDNA3.1-*Ldcen-3*. pCRT7/CT-TOPO-*Ldcen-3*, empty pcDNA3.1, and empty pCRT7/CT-TOPO, PBS was used as a control (chapter 2 methods). The results show that a significant protection was induced by immunisation with 1µg *Ldcen-3* constructs which was vector dependent since pCRT7/CT-TOPO-*Ldcen-3* (4 out of 6 free of lesion) induced better protection than pcDNA3.1-*Ldcen-3* (3 out of 6 free of lesion) (Fig 6.2.4.2-B). The empty pCRT7/CT-TOPO (0/6) vectors did not protect mice from challenge. Although immunisation with empty pcDNA 3.1 vector slowed down lesion development in immunised mice.

A: Immunisation protocol



B:

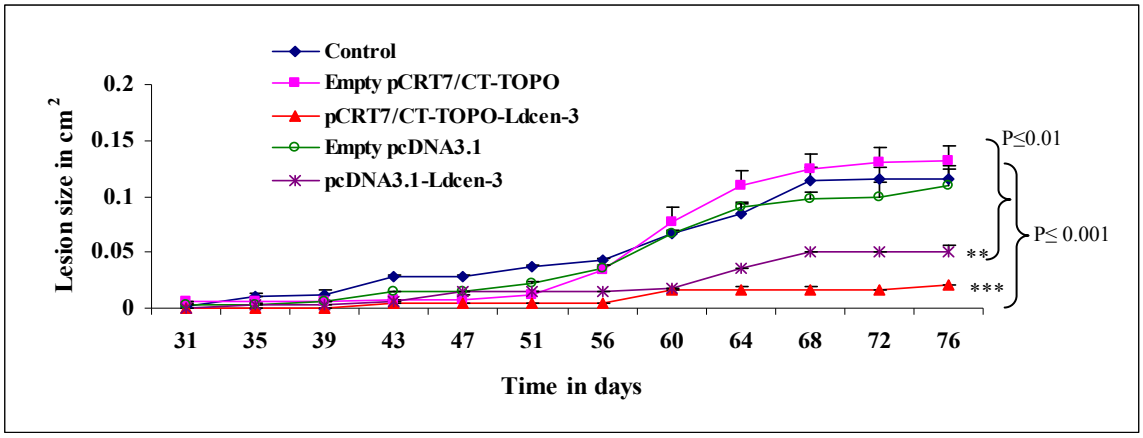
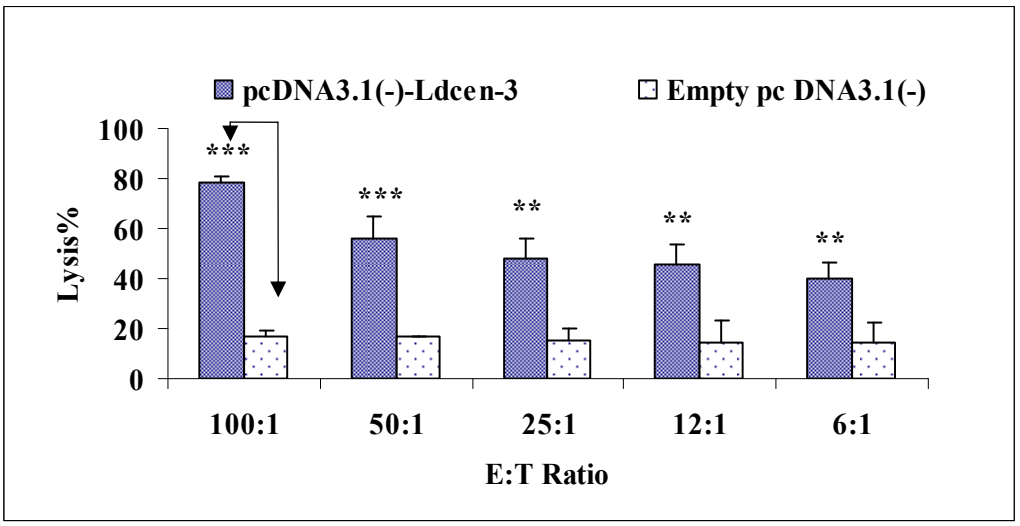


Figure 6.2.4 Immunisation by *Ldcen-3* constructs
A: Immunisation protocol; B: Protection induced by immunisation with pCRT7/CT-TOPO-*Ldcen-3* and pcDNA3.1-*Ldcen-3*: Five groups of Balb/c mice were immunised, with pCRT7/CT-TOPO-*Ldcen-3*, pcDNA3.1 (-) *Ldcen-3*, empty pCRT7/CT-TOPO, empty pcDNA3.1 (-) and PBS by gene gun (1µg/mouse) on days 0 and 14. Seven days after last immunisation mice were challenged with 2×10^6 live *L. mexicana*. Mice were regularly monitored at least twice a week to determine the lesion size on each mouse. The graph represents 2 independent experiments. Bars represent the standard deviation n=6, P**≤0.01 P***≤0.001 by T test.

6.2.5 CTL activity in Balb/c mice immunised with pcDNA3.1 (-)-*Ldcen-3* and pCRT7/CT-TOPO-*Ldcen-3* by gene gun

To evaluate the role of cytotoxic T cells in immunity to *Leishmania*, a standard 4-hour ^{51}Cr-release cytotoxicity assay was used to assess the ability of *L. mexicana Ldcen-3* construct to generate specific cytotoxic T lymphocytes by immunisation. Balb/c mice were immunised with pcDNA3.1 (-)-*Ldcen-3* and pCRT7/CT-TOPO-*Ldcen-3* as previously described in section 6.2.4. Splenocytes were harvested from immunised mice and cultured *in vitro* for 5 days together with blasts cells pulsed with LPS and SLA2 (see chapter 2 methods). On day 5, the splenocyte cells were used as effectors in standard 4-hour ^{51}Cr-release cytotoxicity assay against non-adherent DCs loaded with SLAs and DCs alone as target. Splenocytes from Balb/c mice immunised with pcDNA3.1 (-)-*Ldcen-3* or pCRT7/CT-TOPO-*Ldcen-3* induced significant CTL activity compared with empty vectors (Fig 6.2.5-A&B).

A:



B:

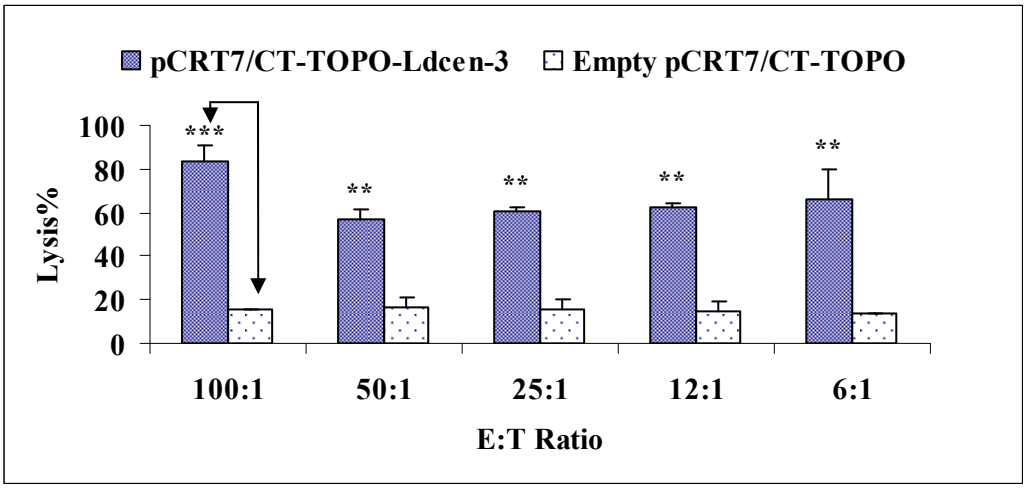


Figure 6.2.5 CTL activity in Balb/c mice immunised with pcDNA3.1 (-)-*Ldcen-3* and pCRT7/CT-TOPO-*Ldcen-3* by gene gun
A: CTL activity of Balb/c mice immunised with 1µg pcDNA3.1-*Ldcen-3* or mice immunised with 1µg empty pcDNA 3.1(-) by gene gun; **B:** CTL activity of Balb/c mice immunised with pCRT7/CT-TOPO-*Ldcen-3* or immunised with empty pCRT7/CT-TOPO by gene gun. Splenocytes were stimulated with SLA for 5 days and used as effector cells in a standard 4-hour cytotoxicity assay against DCs pulsed with SLA.The graph represents 4 mice in 2 independent experiments P**≤0.01, P***≤0.001 by T test.

6.2.6 Transfection of CT26 cells with pcDNA3.1 (-)-*Ldcen-3*

In this study CT26 tumour cells were transfected with pcDNA3.1 (-)-*Ldcen-3* DNA using lipofectamine 2000, according to the manufacturer's instructions, to investigate CTL activity against targets expressing *Ldcen-3* antigen. The presence of the *Ldcen-3* gene was determined in the stable transfected cells by RT-PCR using forward and

reverse primers: *Ldcen-3*F 5`AGA GGC ATT CGT GTT CG-3` and *Ldcen-3*R, reverse 5`AGG TTG ATC TCG CCA TCT TGA -3`. The transfected CT26-*Ldcen-3* clearly shows a strong band for *Ldcen-3*. Also, for unknown reasons, non-transfected CT26 cells always showed a faint band when tested with the primers, which is not a specific band compared with transfected CT26 this was also observed when CT26 cells was transfected with *L. mexicana* gp63 plasmid construct (Ali *et al*., 2009) (Fig 6.2.6).

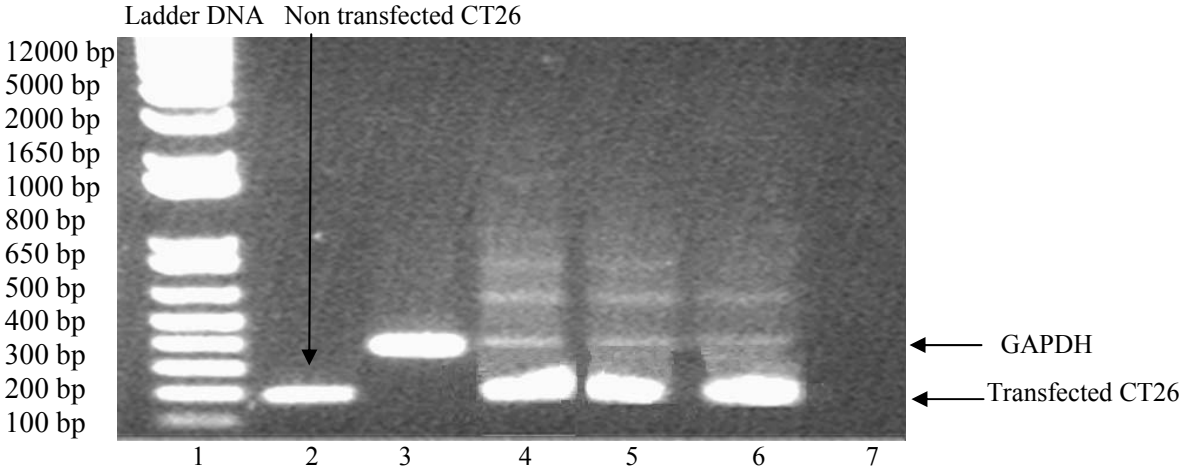


Figure 6.2.6 Expression of *Ldcen-3* gene in transfected CT26 tumour cells as detected by RT-PCR. The expression of *Ldcen-3* in transfected and non-transfected CT26 cells, GAPDH is a mouse house keeping gene used as a positive control. 1: standard DNA 2: non transfected CT26 3: mouse GAPDH, 4, 5, 6 transfected CT26 tumour cells and 7 negative controls.

6.2.6.1 CTL activity in Balb/c mice by immunisation with *Ldcen-3* construct against tumour targets

Balb/c mice were immunised twice at a two week interval with *Ldcen-3* construct coated on gold particles by gene gun. Mice were sacrificed two weeks following the 2nd immunisation and spleens were collected. Splenocytes were harvested and cultured *in vitro* for 5 days together with blasts cells pulsed with LPS and SLA2 (SLA may contain *Ldcen-3* protein). On day 5, the splenocytes cells were used as effectors in a standard 4-hour ^{51}Cr-release cytotoxicity assay against CT26 tumour

cells transfected with *Ldcen-3* (Fig 6.2.6.1). The results clearly show that immunisation of mice with *Ldcen-3* construct induce specific CTL activity against CT26 tumour cells expressing *Ldcen-3*. The *in vitro* restimulation of CTLs by SLA2 loaded blast cells was crucial. It was shown that removing the *in vitro* restimulation of the splenocytes prevented the generation of CTL activity in immunised mice and levels were comparable with that of naïve mouse splenocytes re-stimulated *in vitro* by blast cells loaded with SLA2. Maximum cytotoxity was observed even at the minimum effector to target ratio of 6:1 suggesting the need for further testing with different effector to target ratios for unknown reasons.

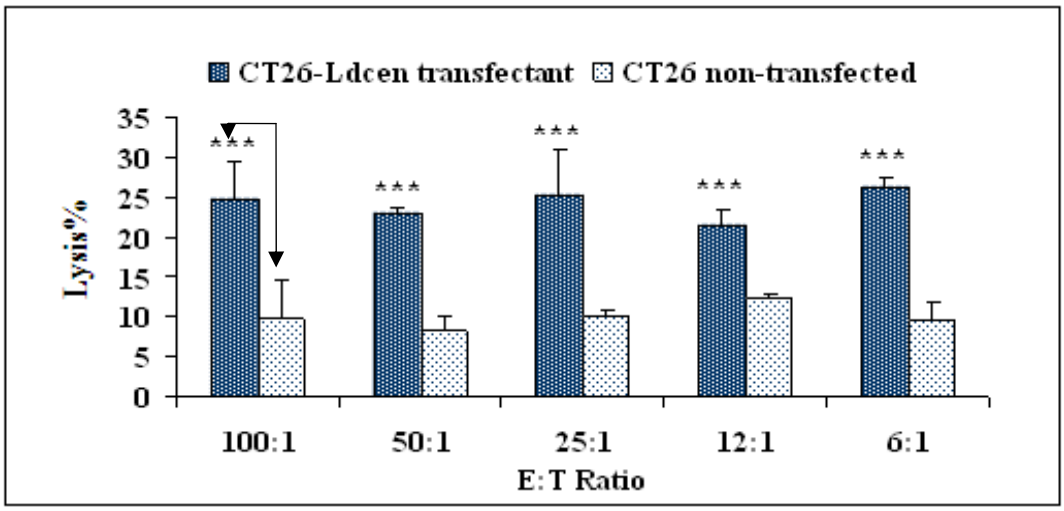


Figure 6.2.6.1 CTL activity of Balb/c mice immunised with 1µg pcDNA-*Ldcen-3*(-) by gene gun
Splenocytes were cultured *in vitro* for 5 days together with blast cells pulsed with LPS and SLA2. On day 5 they were used as effector cells in a standard 4-hour cytotoxicity assay against CT26-*Ldcen-3* and parental CT26. The graph represents 3 independent experiments, P***≤0.001 by T test.

6.3 Discussion

Immunisation with naked plasmid DNA represents a promising new approach in prevention and treatment of various diseases (Ivory and Chadee, 2004). DNA vaccines offer a considerable number of advantages over other vaccines and are therefore an appealing approach to vaccination against Leishmaniasis. A number of studies have demonstrated encouraging results with DNA vaccines and have highlighted their potential in both treatment and protection against Leishmaniasis (Ahmed *et al*., 2004; Dumonteil *et al*., 2003; Kedzierski 2010). DNA vaccines are usually constructed from bacterial plasmids that are designed to express a gene of interest in the host cells to initiate antigen specific immune responses (Spier, 1996; Giri *et al*., 2004). The plasmid DNA enters the cell and goes to the nucleus where it is transcribed to messenger RNA. The transcribed messenger RNA enters the cytoplasm and is translated on the ribosomes. The expressed antigen is presented to corresponding cells and generates a humoral and cell mediated immune response.

There is a homology in the gene sequence of *Ldcen-3* between different species of *Leishmania* (Selvapandiyan *et al*., 2004). *Ldcen-3* appears to be a suitable candidate for a DNA vaccine, since *Ldcen-3* is 100% homologous between *L. donovani, L. mexicana* and *L. major*. Vaccination with plasmid DNA encoding *Ldcen-3* could potentially protect against more than one clinical syndrome in the "Old and New World".

Selvapandiyan *et al*., (2009) have previously shown that immunisation with a live attenuated *L. donovani* centrin 1 gene-deleted parasite (*LdCen1*) could induce significant protection against Leishmaniasis in animals. Balb/c mice immunised with *LdCen1* (*Leishmania* mutant) demonstrated early clearance of virulent parasite

challenge compared with mice immunised with killed parasites, which was associated with a significant increase of cytokine (IFN-γ, IL-2, and TNF) producing $CD4^+$ T cells. Immunised mice also showed increased IgG2a and NO production in macrophages. Balb/c mice immunised with *LdCen1* were cross-protected against *L. braziliensis* suggesting that *LdCen1* is a safe and effective vaccine candidate against visceral and mucocutaneous Leishmaniasis.

To transfect CT26 tumour cells with *Ldcen-3* to be used as targets to assess CTL activities in Balb/c mice immunised with *Ldcen-3* plasmid construct, it was decided to sub clone the *Ldcen-3* from pCRT7/CT-TOPO into a known mammalian pcDNA 3.1 plasmid. Garmory *et al*., (2003) have reported that pcDNA 3.1 is a suitable mammalian vector having the cytomegalovirus (CMV) promoter which is required for optimal expression in mammalian cells. Also, pcDNA3.1/hygro is a suitable vector for a DNA vaccine. pcDNA3, which is very similar to pcDNA3.1/Hygro, has been used in other studies as a back bone for DNA vaccines against Leishmaniasis (Ghosh *et al*., 2002; Mendez *et al*., 2002). Therefore, *Ldcen-3* was sub cloned from pCRT7/CT-TOPO into pcDNA 3.1 to be used as a vaccine and also to be transfected into CT26 tumour cell to be used as target cells in CTL assays.

CT26 transfected with *Leishmania* centrin is expected to present centrin-3 antigen on their surface MHC I and would be a suitable target for CTL activity against *Leishmania* antigens. Stable transfectants expressing *Leishmania* antigens would provide a suitable alternative target to fresh DCs in cytotoxicity assays. Splenocytes from Balb/c mice immunised with pcDNA3.1-*Ldcen-3* or pCRT7/CT-TOPO-*Ldcen-3* induced a potent CTL response compared to the control group against either DC

targets loaded with SLA2 or CT26 tumour cells expressing *Ldcen-3*. Tumour cells can act as professional APC that would specially generate CTL if they express a tumor peptide-MHC class I complex and co-stimulatory molecules (Conry *et al*., 1996; Sarobe *et al*., 2004). The immunogenicity of *Ldcen-3* cDNA cloned in the pCRT7/CT-TOPO plasmid and pcDNA3.1 was determined via DNA vaccination in a Balb/c mouse model *in vivo*. Mice immunised with *Ldcen-3* in pCRT7/CT-TOPO or in pcDNA3.1 (-) were significantly protected against challenge with live parasites; the known immunogenicity gp63 gene was used as a positive control. A dominant Th1 response was shown to have been correlated with protection in several animal models for *Leishmania* infection. Immunisation of Balb/c mice with a plasmid DNA vaccine containing gp63 gene from *L. major,* induced a dominant Th1 response that was protective against challenges with live parasites *in vivo* (Xu & Liew, 1995; Ali *et al*., 2009). Susceptibility of Balb/c mice to *Leishmania major* infection was correlated to an inability to generate a Th1 response which could be restored by administration of IL-12 (Trinchieri, 1995; Barbi *et al*., 2008). This Th response would aid the development of $CD8^+$ CTLs capable of killing cells expressing appropriate antigen.

DNA vaccines produce potent CD8 CTL responses in mice against antigens from parasites and tumours. The construction of DNA vaccine-encoded antigens able to produce a CTL response includes whole protein, truncated protein and fusion with another protein (Horspool *et al*., 1998; Morcock *et al*., 2000). Conry *et al*., (1996) and Jacobsen *et al*., (2007) have found that if the tumour cells are tranfected with plasmid DNA containing a tumour antigen gene then a specific CTL may be generated. In this work (Fig 6.2.6.1) CT26 tumour cells transfected with pcDNA3-*Ldcen-3* were shown to be susceptible target cells to CTLs derived from Balb/c mice immunised with

pcDNA3-*Ldcen-3* by gene gun. Ali *et al*., (2009) have demonstrated a potent CTL activity in cultured splenocytes from Balb/c mice immunised *L. mexicana* gp63 DNA plasmid using I.M. injection and gene gun immunisation with against CT26 tumour cells transfected with pcDNA-gp63. Qin *et al*., (2010) have shown a method of DNA immunisation using a prime-boost immunisation strategy (two different vaccines, each encoding the same antigen, given several weeks apart); better protection was obtained by gene gun immunisation. In addition Gurunathan *et al*., (1998) have reported the presence of long term antigen-specific Th1 activity in mice immunised with a DNA vaccine containing a gene that coded for a *Leishmania* antigen. Rodriguez-Cortes *et al*., (2007) found that a multivalent DNA vaccine, encoding TRYP which is a key enzyme of the trypanothione dependent metabolism for removal of oxidative stress in *Leishmania*, LACK and gp63, did not protect dogs against *L. infantum* experimental challenge, inspite of the hypothesis that an effective immune response was more likely to be generated following exposure to more than one antigen. Alternatively, Carter *et al*., (2007) established that Balb/c mice immunised intramuscularly by parasite enzyme gammaglutamylcysteine synthetase DNA vaccine protected them against *L. donovani*.

This study has shown for first time that Balb/c mice immunised with pcDNA 3.1-*Ldcen-3* or pCRT7/CT-TOPO *Ldcen-3* constructs by gene gun induced potent protection against challenge with *L. mexicana* which was also correlated with CTL activity.

Chapter 7 Discussion

7.1 Discussion

Existing vaccine strategies including the use of whole cell lysate, killed parasite and recombinant antigens, some degree of effectiveness in animal models but achieved little or no protection in humans. Therefore the search for new antigens and new vaccine strategies is an ongoing process. Parasite persistence was found to be important for an effective protective response that could be achieved naturally or by immunisation with live attenuated or mutant parasite (Selvapandiyan *et al*., 2006 and Carvalho *et al*., 2010). DNA vaccine is one of the most novel ways that can be used to develop vaccines against Leishmaniasis. DNA vaccines can also be used to raise all types of immune response including T-helper cells, a crucial step for an effective immune response against intracellular parasites (Dumonteil, 2007; Choudhury, 2010).

In the present study cell-mediated immune responses were evaluated in Balb/c mice by measuring T-cell proliferation, cytokine production, and phenotypic characterisation of splenocyte T-cell populations. Two types of SLA antigens; SLA1 and SLA2, were produced by two different methods. The main difference between SLA 1&2 was: for SLA1 antigen, parasites were lysed by sonication for 45 minutes followed by ultracentrifugation of the lysate for 4 hours at 100,000g, while SLA2 antigen was produced by sonication of the parasites for 2 minutes without ultracentrifugation. Comparable immune responses to immunisation with SLA1 and SLA2 were observed against *Leishmania* infections. Immunisation with SLA1 or SLA2 significantly protected mice against challenge with live *L. mexicana*. However, immunisation with DCs loaded with SLA did not protect mice against challenge with live *L. mexicana* in spite of the activation of CTL activity as determined by ^{51}Cr release cytotoxicty assay.

Similar responses to *L. major* promastigote SLA have been reported by Scott *et al*., (1987a) and Badiee *et al*., (2007) where the intraperitoneal immunisation of Balb/c mice with *L. major* promastigote SLA induced significant protection against *L. major* infection. It has been revealed that the immunity induced by this vaccine was associated with the generation of cell-mediated immunity which did not require the development of an antibody response to promastigote surface antigens. Similar results were also reported by Ali *et al*., (2009) where Balb/c mice immunised with SLA induced potent CTL activity against DC loaded with SLA. Most of the published studies did not correlate protection against challenge with live parasite with CTL activities in the immunised mice since only a few studies have measured CTL activities in immunised animals (Bhowmick *et al*., 2010). The poor protection induced by immunisation with DCs loaded with SLA reported in this study could not be fully explained since potent CTLs were detected in spleens of the immunised mice.

Since soluble *Leishmania* antigen has induced effective protection against *L. mexicana* infection in Balb/c mice, it was decided to analyse in more depth the component of this Ag and whether it is possible to isolate a highly immunogenic single Ag.

To identify and characterise the immunogenicity and immune responses to components of the SLA antigens, SLA2 was separated into a number of fractions. SLA2 was fractionated into six sub fractions by FPLC. Fractions of SLA2 were separated according to anion exchange by a Mono Q column in order to study the ability of each fraction to stimulate T cells by immunisation of Balb/c mice. Gp63 protein, a well characterised *Leishmania* protein, was used as a marker and a target antigen for a comparative immunological analysis since gp63 was the antigen of choice in many vaccine studies (Sachdeva *et al*., 2010). According to the western-

blotting analysis, a strong gp63 band was detected in whole SLA1&2 and much less in sub fractions 2 and 3, but not in sub fractions 1, 4, 5 and 6. These results suggest that this separation technique has failed to concentrate at least this particular protein gp63. Immune responses to immunisation with these antigens were measured *in vivo* and *in vitro* by challenging immunised mice with live parasites and CTL assay respectively. Splenocytes from Balb/c mice immunised with *L. mexicana* SLA2 or SLA2 fractions in combination with IFA induced a potent CTL response compared to the control group, but the whole unfractionated SLA2 induced the highest CTL activity compared with all other fractions. These experiments have also showed that IFA adjuvant alone did not stimulate specific anti SLA responses since mixing Incomplete Freund's Adjuvant with poor immunogenic fractions did not induce significant responses. This observation led to postulating that no single fraction was superior in antigenicity to whole SLA. In a study by Scott *et al*., (1987b) and Vilela *et al*., (2007) Balb/c mice immunised with *L. major* SLA in combination with *Corynebacterium parvum* (CP) adjuvant, induced high protection against challenge with *L. major*. *L. major* SLA was further fractionated into nine sub-fractions by anion exchange liquid chromatography and investigated for their ability to stimulate T cells in immunised Balb/c mice. Two fractions (fractions 1 and 9) only were able to stimulate lymphocytes to produce macrophage-activating factor as measured by a macrophage *Leishmaniacidal* assay, and elicited significant delayed-type hypersensitivity though the study did not look at CTL responses. It has been reported that immunisation of Balb/c mice with SLA in IFA plus Ad5IL-12 (administration of an adenovirus expressing IL-12) induced protection against *L. major* as measured by decreases in lesion size (Gabaglia *et al*., 2004).

The results in this study clearly show that immunisation with SLA1&2 prevented the development of lesions in susceptible Balb/c mice and the observed protection could be due to the development of Th1 responses. In agreement with results presented in this study, Trotta *et al*., (2010) have shown that macrophages from dogs vaccinated with *L. infantum* promastigote soluble antigen formulated with three different adjuvants were able to kill *Leishmania* parasite *in vitro* for up to 12 months after vaccination suggesting potency of this vaccine in dogs. Stacey & Blackwell, (1999) have earlier reported that subcutaneous immunisation of Balb/c mice with SLA alone, or SLA plus CpG ODN induced significant protection, but mice that received SLA plus CPG oligodeoxynucleotide (CpG ODN) had significantly less lesion size compared with those receiving SLA alone. Ramirez *et al*., (2010) also have shown that immunisation with ribosomal protein extracts administered in combination with CpG oligodeoxynucleotides protects susceptible Balb/c mice against primary *L. major* infection.

The ability of each SLA2 fraction to stimulate T cells from immunised Balb/c mice *in vitro* was determined by a cell proliferation assay. Significant proliferation was noticed in splenocytes stimulated with SLA2 and SLA2 fractions, but DCs loaded with SLA2 or SLA2 fractions induced much potent responses compared with SLA2 or SLA2 fractions alone. It was also noticed that splenocyte proliferation responses to whole SLA2, fr1 and fr2 was higher than that of fr3, 4, 5 and 6. Ajdary *et al*., (2000, 2009) have compared proliferation responses of PBMCs to *L. major* antigen between two groups of subjects, demonstrating that PBMCs from patients with active lesions (newly infected) responded strongly to SLA, but not that of the non-healing patients (chronic infection).

Additionally, *in vitro* stimulation of splenocytes from immunised Balb/c mice with whole SLA2 induced significant levels of IL-2 compared with stimulation with fraction fr1, fr2 and fr3, whereas high levels of IFN-γ and IL-12 were detected in supernatants of both naïve and immune splenocytes. No or low levels of IL-4 were detected in response to *in vitro* stimulation with SLA. Ajdary *et al*., (2000 & 2009) have found similar results, PBMC from *Leishmania* patients with active lesions produced high levels of IFN-γ and no or little IL-4 in response to *in vitro* stimulation with SLA, but no or low levels of IFN-γ and high levels of IL-4 production in non-healing patients suggesting the presence of Th1 and Th2 responses, respectively. PBMC from normal controls did not produce significant levels of IFN-γ or IL-4. In addition Park *et al*., (2002) and Cumming *et al*., (2010) have shown that resistance of C57BL/6 mice to *Leishmania* infection was due to the activation of a Th1 type response and IFN-γ production. This response has been shown to be initiated by IL-12 activation which was subsequently followed by activation of macrophages and NK cells. Balb/c mice on the other hand had a Th2 response to *Leishmania* infection which was associated with the production of IL-4 which in turn suppresses the receptors for IL-12 and ultimately leads to susceptibility to this parasite (Jones *et al*., 1998; Lapara & Kelly, 2010). The control of *L. infantum* in C57BL/6 and Balb/c mice was shown to be dependent on CD4 T cell activation and IL-12 production which stimulates IFN-γ production from CD8 T cells and NK cells (Murray, 1997; Beattie et al., 2010). Balb/c mice immunised with different concentrations of ScLL (Synadenium carinatum latex lectin) associated with SLA induced a high level of protection against cutaneous Leishmaniasis, and high levels of IgG2a and an

increased expression of mRNA for IL-12, IFN-γ and TFN-α (Afonso-Cardoso *et al*., 2007).

IgG2a and IgG1 antibody response profiles (antibody isotypes) in this study have revealed a complex Th1/Th2 response to SLA1 and SLA2 immunisation, since increased levels of IgG2a and IgG1 in the serum of mice immunised with SLA1 and SLA2 as early as 7 days after the immunisation was demonstrated. The antibody responses against SLA2 in the first 2 weeks were higher than those against SLA1. Similar results were also reported by Ramirez (2010), Balb/c mice immunised with *L. major* ribosomal protein (LRP) extracts administered in combination with CpG oligodeoxynucleotides, induced long-term Th1 dependent protection against *L. major* secondary infection. Also, cells cultured from lymph node and spleens produced high levels of IFN-*γ* but not IL-10 and IL-4. High levels of IgG2a and IgG1 similar to results reported in this study were also detected in immunised mice. In addition Mohammadi *et al*., (2006) have identified two novel *L. major* promastigote antigens (140 and 152 kDa) that are able to induce a specific IgG2a response in C57BL/6 and Balb/c mice. These two proteins were also shown to induce IgG production in mice. A single subcutaneous or intraperitoneal injection of mice with *L. mexicana* antigens induced both Th1 and Th2 responses as determined by IgG1 and IgG2a antibodies (Dissanayake *et al*., 2005; Bhowmick & Ali, 2009). Balb/c mice immunised with SLA encased within Man5-DPPE (dipalmitol phosphatidy lethanol- amine) induced a potent protective response to *L. major* infection and increased IgG2a/IgG1 levels in the sera of immunised mice (Shimizu *et al*., 2003). Collectively results presented in this study suggest that the immunogenicity of the whole SLA is much superior to any of the single SLA fractions.

DC subsets may have different functions in terms of Th1/Th2 generation, antigen recognition and cytokine production (Sundquist *et al*., 2004; Nylen & Gautam, 2010). DC subsets of murine spleen may differ in their ability to generate IL-12, a potent initiator of anti *Leishmania* responses and phagocytosis of the *Leishmania* parasite (Henri *et al*., 2002; Steinman & Idoyaga, 2010). The function of B and T lymphocytes, as mediators of immunity are largely under the control of dendritic cells, DCs residing in the periphery take up and process antigens, express co-stimulatory molecules, migrate to lymphoid organs, secrete cytokines and interact with lymphocytes to initiate an immune response. Down regulation of MHC class I and II expression on infected DCs may be used as an immune evasion strategy by the *Leishmania* parasite. It has been shown that mature activated antigen-containing DCs display high levels of MHC class I, MHC class II and co-stimulatory molecules on their cell surfaces, which leads to activation of T cells (Meyer *et al*., 2004). This study was focused on the effect of live and autoclaved *L. mexicana* on the expression of MHC I, MHC II, CD40, CD80 and CD11c on the surface of DCs derived from the bone marrow of Balb/c mice and whether down regulation of MHC class I reduced the of infected cells to CTL killing. In order to establish the phenotypic profile of DCs, different types of bone marrow derived cells i.e. mature adherent, immature adherent, mature non-adherent and immature non-adherent cells were stained with different DC markers and analysed by flow cytometer. Only mature non-adherent cells expressed all potential DC markers: such as MHC I, MHC II, CD11c, CD205, CD40 and CD80, but did not express the macrophage marker F4/80. These results clearly indicated that only mature non-adherent cells have the highest percentage of DCs, hence they were chosen for further studies. Maturation of DCs was shown to increase the expression of MHC I & Class II and co-stimulatory molecules such as

CD40, CD80, CD86 and CD54, and also down-regulated their antigen capturing and phagocytic capacity (Brandonisio *et al*., 2004; Yildirim *et al*., 2010). On the other hand, maturation of DCs enhanced cytokine secretion, induced different patterns of chemokine receptor expression and chemokine production, enabling DC migration and recruitment of other cell types. Infection of DCs for one or 24 hours with live *L. mexicana* parasite down regulated the expression of MHC I, MHC II, CD40, CD80 and CD11c. Brandonisio *et al*., (2004) and Young *et al.*, (2010) have found that DCs in humans and mice can be divided into various subtypes dependent on the type of surface antigens. On the basis of the expression of CD4, CD8, CD205 and CD11b surface markers, myeloid and plasmocytoid DC subsets in humans and mice have shown partially diverse functions (Henri *et al*., 2002; Comabella *et al*., 2010). Results in this study demonstrate that BM DCs of Balb/c mice express high levels of $CD11c^{+}$ and infection with live *L. mexicana* induced down-regulation of MHC I, MHC II, CD80, CD40 and CD11c. Similar results were reported by (Muraille *et al*., (2003) and Griewank *et al*., (2010) where infection of CD11c+ DC with *L. major* decreased the expression levels of MHC II. Unlike infection with live parasites, treatment of DCs with autoclaved parasites did not down regulate, but slightly up-regulated the expression of MHC I, MHC II, CD40, CD80 and CD11c and is in contrast to the effect of both infective and lysed parasites on CCR2, CCR3 & CCR7 expression by DCs (Steigerwald & Moll, 2005; Jimenez *et al*., 2010).

The combination of a therapeutic vaccine with antibiotic was found to be more effective in curing the lesions of bacterial infections in animal models (Kedzierski 2010). Based on this hypothesis, experiments were designed to study the effect of treatment with fungizone on the expression of MHC I, MHC II, CD40, CD80 and CD11c by DCs following *Leishmania* infection. It has been postulated that down

regulation of MHC I and II on the surface of *Leishmania* infected DCs would help the survival of the parasite inside the target cells despite the presence of potent CTLs, thus leading to progression of infection. If the down regulation of MHCs can be reversed by the use of fungizone, the combination of vaccine and fungizone could be effectively used to treat *Leishmania* infection. No toxic effect of fungizone on DCs was observed, as determined by culturing DCs with increased doses of fungizone for 24 hrs. The addition of fungizone to DC cultures for one hour subsequent to *Leishmania* infection up-regulated to normal levels the expression of MHC class I, MHC class II, CD11c, CD80 and CD40. This also has restored their susceptibility to CTL activity. DCs loaded with autoclaved *L. mexicana* were found to be susceptible to CTL killing which was positively correlated with MHC class I expression, but DCs infected with live parasites showed decreased levels of surface expression of MHC I and subsequently decreased their susceptibility to CTL activity suggesting a possible mechanism used by the parasite to evade immunity.

DC responses to *Leishmania* infection are varied according to the genetic background of the mice which determine their resistance or susceptibility to *Leishmania* infection. Skin DCs from *L. major* infected C57BL/6 (resistant) or susceptible Balb/c mice both demonstrate up-regulation of MHC class I and II, CD40, CD54, and CD86 and release IL-12p70 (von Stebut *et al*., 2000; Griewank *et al*., 2010). IL-4R expression was high in LCs infected with *L. major* from susceptible but not resistant mice (Moll *et al*., 2002) and the co-stimulatory molecule CD80 expression was down-regulated on LCs from susceptible but not resistant mice (Mbow *et al*., 2001). Furthermore, CD40 expression was decreased in the lymph node DCs of susceptible mice infected *in vivo* with *L. major*, but not of resistant mice, which was also correlated with under-

production of IL-12p40 and IL-12p40 mRNA (Heinzel *et al*., 1998; Teixeira *et al.*, 2010). Collectively these results suggest the presence of more than one factor that controls susceptibility or resistance to *Leishmania* infection. Also, these results suggest the potential efficacy of combination treatment of *Leishmania.*

Plasmid DNA encoding single or multiple proteins from pathogens when introduced into host cells by immunisation promotes humoral and cell-mediated immune responses (Walker *et al*., 1998; Moreno & Timon, 2004). It has been reported that immunisation with a DNA plasmid encoding one or more *Leishmania* antigens produced protection against the parasite (Kedzierski, 2010). In Balb/c mice, injection of *L. major* gp63 plasmid by gene gun DNA induced high protection against *Leishmania* infection, where IFN-γ but not IL-4 was produced by immune T cells (Ali *et al*., 2009). Immune responses induced by plasmid DNA vaccines can be moderated by means of administration and/or the co-administration of immuno-modulator genes; this has been well established in animal studies (Ertl, 2009). Several differences in responses to DNA vaccination between mice and humans have already been observed. In humans, high doses of plasmid DNA are required to induce an immune response but in mice, similar responses are normally obtained using 0.1-1μg of DNA administered by a gene gun and 10-100μg administered by injection (Fynan *et al*., 1993; Roth *et al*., 2006). Balb/c mice immunised with a DNA vaccine of *L. amazonensis* gene constructs encoding P4 nuclease, *L. amazonensis* HSP70 and adjuvant constructs encoding murine IL-12, induced significant protection against Old World and New World cutaneous Leishmaniasis (Campbell *et al*., 2003; Shaddel *et al*., 2008; de Oliveira *et al*., 2009).

In the present study the immunogenicity of *Ldcen-3* was determined using DNA immunisation in a Balb/c mouse model of the *L. mexicania* parasite. The *Ldcen3* gene

is 100% homologous in *L. major, L. donovani,* and *L. mexicana.* Thus, vaccination with plasmid encoding *Ldcen-3* could potentially protect against more than one Old and New World clinical syndrome. *Ldcen-3* gene was originally constructed in pCRT7/CT-TOPO (Selvapandiyan *et al*., 2006, 2009), but it was not known whether it could be expressed in mammalian cells, to determine whether pCRT7/CT-TOPO is a suitable vector for use in mammals, a marker gene (*LacZ*) was cloned in this plasmid and used to transfect the mouse cell line CT26. In this study, immunisation of Balb/c mice with *Ldcen-3* cDNA by gene gun induced specific CTL activity against CT26 tumour cells expressing *Ldcen-3* was it tested against DCs loaded with SLA as well. It was shown that Balb/c mice immunised with pcDNA 3.1-*Ldcen-3* or pCRT7/CT-TOPO constructs by gene gun induced potent protection against challenge with *L. mexicana* which was also correlated with CTL activity. In a previous study by Dumonteil *et al*., (2003) Balb/c mice immunised with DNA vaccines encoding *L. mexicana* GP63, LACK and CPb in VR1012 induced protection against *L. mexicana* as demonstrated by reduced lesion size and parasite burden. Immunisation of mice with a mixture of these three plasmids also increased the protection. The results presented in this study demonstrated for the first time the immunogenicity of the *Ldcen-3* gene and its potential as a vaccine candidate for further investigation.

Future Work

- Centrin-3 is homologous in *L. donovani*, *L. major*, and *L .mexicana* therefore, it is hopeful that this vaccine could confer protection against all three species. However, the immune evasion strategies employed by the parasite vary between species. Therefore, it would be very interesting to conduct a study to investigate the ability of pcDNA3.1- *Ldcen3* to protect against infection with each of these three species.

- *Ldcen-3* was sub-cloned successfully from pCRT7/CT-TOPO into pcDNA 3.1. It was shown that immunisation of Balb/c mice with pcDNA 3.1-*Ldcen-3* and pCRT7/CT-TOPO constructs by gene gun induced potent protection against challenge with *L. mexicana*, although for unknown reasons better protection was afforded by pCRT7/CT-TOPO-*Ldcen-3* vector immunisation. Based upon these findings, further work should be undertaken using the three *Leishmania* genes (gp63, *Ldcen-1, Ldcen-3*.) cloned into different vectors (VR1012, pCR T7/CT-TOPO and pc DNA 3.1), to investigate their ability in protection against *Leishmania* infection in a combination. This could result in a novel and more effective vaccination strategy against this disease.

- In this study, DCs infected with live parasites was shown to have decreased levels of surface expression of MHC I and subsequently decreased their susceptibility to CTL activity suggesting a possible mechanism used by the parasite to evade immunity. *Leishmania* parasites were shown to lose their infectivity (virulence) on prolonged culturing *in vitro*. Virulent and avirulent parasite cultures will be further investigated for their ability to infect

macrophage and whether they are able to affect the expression of MHC class I, II, CD11c, CD40 and CD80 in a similar manner.

Appendix I

Materials

Laboratory Plastics, Glassware and Sharps

Instrument	Manufacturer
PCR tubes	Micronic Systems
Conical Flask	Pyrex
Measuring cylinder	Kartell®
10µl Micropipettes	Sarstedt, UK
100µl Micropipettes	Sarstedt, UK
1000µl Micropipettes	Sarstedt, UK
Micro tips 0.5 – 10µl tips	Sarstedt, UK
Micro tips 20 – 200µl tips	Sarstedt, UK
Micro tips 200 – 1000µl tips	Sarstedt, UK
96 well ELISA plates	Costar, UK
Petri dishes	Sterilin UK
25 ml Pipettes	Sarstedt, UK
24 well and 6 well flat bottom culture dishes	Sarstedt, UK
96 well round bottom plates	Sarstedt, UK
Pasteur pipettes	Sarstedt, UK
1.5 ml eppendorf tubes	Sarstedt, UK
0.5 ml eppendorf tubes	Sarstedt, UK
1.2 ml Cryovials	TPP, UK
Pipette tips < 1ml	Sarstedt, UK
96 well ELISA plates	Costar, UK
Petri dishes	Sterilin UK
25 ml Pipettes	Sarstedt, UK
10 ml Pipettes	Sarstedt, UK
5 ml Pipettes	Sarstedt, UK
Haemocytometer	Weber

96 well plate harvester filters	Perkin Elmer
Scalpels	Swann Morton Ltd.
PCR Tubes	Micronic Systems
0.2µm Filters	Sartorius, UK
Real-time PCR tubes	Strategene, Germany
0.5 – 10µl tips	Sarstedt, UK
20 – 200µl tips	Sarstedt, UK
200 – 1000µl tips	Sarstedt, UK
Dialysis tube	Sarstedt, UK
FACS Tubes	Sarstedt, UK

Electrical Equipment

Instrument	Manufacturer
Liquid Nitrogen Freezer	Forma Scientific
-80°C Freezer	Ultima II, Revco
Class II safety cabinets	Walker
37°C incubator	Forma Scientific
96 well plate harvester	Packard
Light microscope	Olympus
96 well plate reader	Tecan
Top count scintillation counter	Packard
Drying Cabinet	Scientific Laboratory Supplies Ltd
PCR Thermal Cycler	Hybaid, Germany
Water Baths	Grant Instruments
Real Time PCR Thermal Cycler	Bio-rad
Microscope	Nikon
Power Packs	Bio-rad
UV Spectrophotometer	Sanyo
Tran illuminator	Ultra Violet Products
Whirl mixer	Scientific Industries
Coulter Flow Centre	Beckman Coulter
-80°C Freezer	Ultima II, Revco
-20°C Freezer	Standinova
Orbital Incubator	Stuart
Incubator	Griffin
Electrophoresis gel tanks	Bio-rad
Power Pack	Bio-rad
Microwave	Matsui
Nanopure Diamond water reservoir	Barnstead
SP Bio Spectrophotometer	Sanyo

Weighing scale	Fisherbrand
Gene Genius Bioimaging system	Syngene
Whirlimixer	Scientific Industries
PCR Thermal Cycler	Hybaid, Germany
Refrigerated centrifuge	Mistral 1000, MSE
Clenz	Beckman Coulter
Isoton	Beckman Coulter
Flow-chek™ Fluorospheres	Beckman Coulter

Reagents, Bulking

Reagent	Company
Bacterial culture *Escherichia coli* XLIB	Invitrogen
pCR®II- T7/CT-TOPO® 4.0kb	Invitrogen Ltd.
pcDNA3.1/Hygro 5.6kb	Invitrogen Ltd.
pcDNA3.1myc-His LacZ (-) 8.6 kb	Invitrogen Ltd.
V1012 gp63	Invitrogen Ltd.
Sodium chloride	Sigma®, UK
Ampicillin	Sigma®, UK
Kanamycin	Sigma
GTE	Sigma Aldrich, UK
EDTA (Ethylene Damien Tera Acetic Acid)	Sigma Aldrich, UK
HCL	Sigma Aldrich, UK
Tris	Sigma Aldrich, UK
Glucose	Sigma Aldrich, UK
SDS (sodium dodecyl sulphate)	Sigma Aldrich, UK
Sodium hydroxide	Fisher Scientific Ltd.
Potassium acetate	Sigma Aldrich
Acetic Acid	Fisher Scientific Ltd
Chloroform alcohol	Sigma Aldrich
Absolute ethanol	BDH
RNAase	Sigma®
LB Agar	
7.5 gm Agarose	Bioline
5 gmTryptone	Oxoid
2.5 gmYeast	Oxoid
5 gm Sodium Chloride	Sigma
500 ml distilled water	
LB broth	
10g Tryptone	Oxoid
5 g yeast Extract	Oxoid
10g NaCl	Sigma
15g Agar	Bioline
1ml of 1M NaOH (1M = 0.4g/10ml)	Sigma
Add water to 1L and autoclave.	

Agarose gel Electrophoresis	
Agarose	Bioline
Sybr® Safe DNA gel stain	Invitrogen Ltd.
Ethidium Bromide	Sigma
Orange G	Sigma®
DNA Ladder	Invitrogen Ltd.

Polymerase Chain Reaction (PCR)

10x Reaction Buffer	Promega, USA
Magnesium chloride	Promega, USA
Deoxy nucleotide triphosphates (dNTP)	Bioline
Taq Polymerase	Bioline
Primers	MWG Biotech, UK

Restriction Digestion

Bovine Serum Albumin (BSA)	Promega, USA
Buffer B	Promega, USA
Hind III Restriction Enzyme	Promega, USA
EcoR I	Promega, USA

DNA Extraction

Buffer EB	Qiagen
Buffer PE	Qiagen
Buffer QG	Qiagen
Absolute Ethanol	BDH
Isopropanol	Sigma

Ligation Reaction

T4 DNA Ligase	Promega, USA
Ligase Buffer	Promega, USA

Coating of gold particles by DNA

Spermidine	Sigma
Nitrogen gas	Sigma
Gold	BIO-RAD
poly-vinyl-pyrollidone (PVP)	Sigma
Magnesium chloride	Promega

RT-PCR Enzymes and Reagents

Reagent	Company
Oligo dT Primers	Promega
RNasin (Ribonuclease inhibitor;40U/µl)	Promega
M-MLV Reverse Transcriptase (M-MLV RT ; 200U/ml)	Promega
BioTaq Polymerase	Bioline
T4 Ligase Enzyme	Promega
GAPDH (primers)	MWG Biotech, UK
Centrin-3 primers	MWG Biotech, UK
Phusion Taq polymerase	Finnzyme
pcDNA3 plasmid	Invitrogen
SYBR Green Master Mix	Biorad
dNTPs	Bioline
DNA ladder (1Kb plus)	Invitrogen
10x Reaction Buffer	Promega
Magnesium Chloride	Promega
Molecular Grade Water	Sigma, UK
Absolut Ethanol	BDH, UK
Isopropanol	Sigma, UK
RNA Stat 60	AMS Biotechnology, UK
Chloroform alcohol	Sigma Aldrich

Other Reagents

Media

Culture Media	Company
DMEM	Bio Whittaker, Europe
RPMI 1640	Bio Whittaker, Europe
Schneider	Sigma

Supplements added to Culture

Media	Company
Foetal Calf Serum (FCS)	Bio Whittaker, Europe
Glutamine synthetase (GS) and 10%	IRH Biosciences
Bio-FCS (FCS without bovine IgG)	Autogen Bioclear, UK Ltd.
2-mercaptoethanol	Bio Whittaker, Europe
Penicillin/Streptomycin	Bio Whittaker, Europe
HEPES buffer	Bio Whittaker, Europe
Fungizone	Bio Whittaker, Europe
Geniticin (G418)	Bio Whittaker, Europe

Appendix II

T cell Media

Ingredients	Quantity
Complete RPMI	500 ml
10% FCS (by volume)	50 ml
Glutamine	5 ml
20 mM HEPES	10 ml
50 µM 2 Mercaptoethanol	500 µl
50U/ml Penicillin/Streptomycin	5 ml
0.25 µg/ml Fungizone	500 µl

BM-DC media

Ingredients	Quantity
Complete RPMI	500 ml
10% FCS (by volume)	25 ml
Glutamine	5 ml
20 mM HEPES	10 ml
50µM 2 Mercaptoethanol	500 µl
50U/ml Penicillin/Streptomycin	5 ml
0.25 µg/ml Fungizone	500 µl

Schneider media

Ingredients	Quantity
Complete Schneider's Drosophila Media	1000 ml
10% FCS (by volume)	100 ml

PBS
0.15 M NaCl
0.01 M sodium phosphate, pH 7.2

Other Reagents

Reagent	Company
EMLA Anaesthetic Cream	Astra Zeneca, UK
Chromium 51	Amersham
Incomplete Freunds adjuvant (IFA)	Gibco, UK
Trypsin	Gibco, UK
Versene	Gibco, UK
Heparin	Sigma, UK
Trypan Blue	Sigma, UK
Trypan Blue	Sigma, UK
X-gal solution	Sigma, UK

PBS-BSA washes for flow cytometry

Ingredients	Quantity
PBS tablets	10/litre
BSA	0.1% (1g/litre)
Sodium Azide	0.02% (0.2 g/litre)

RIP Buffer

Reagent	gm/500 ml	mM
Sodium Chloride	4.38	150
Tris	3.027	50
EDTA, anhydrous	0.931	5

Western Blot Lysis Buffer

Ingredients	Quantity
RIP Buffer	5 ml
Igepal	50 µl
Deoxycholate acid	25 mg
10% SDS	50 µl
500 mM Benzamidine	10 µl
100 mM PMSF	5 µl
200 mM Sodium Valrpoate	25 µl
1 M Sodium Fluoride	5 µl
Standard protein ladder (10-200 kDa)	Invitrogen

Resolving gel	10%
1165µl acrylamide,	1165µl
Tris	875µl
1460µl H2O,	1460µl
ammonium persulphate	35µl

Atacking gel	4%
Acrylamide	15%
0.5 M Tris HCL	25%
dH_20	60%
Ammonium persulphate	10%
TEMED	0.1%

Other Buffer

Buffer	Composition
PBS	1 tablet dissolved in 100 ml distilled water
PBA	PBS, 0.1% (w/v) BSA, 0.02% (w/v) Sodium Azide
TBS	10mM Tris, 150nM NaCl pH 7.4
1 x TAE (Freshly prepared from 10 x TAE)	40 mM Tris Acetate, 1 mM EDTA

***Leishmania* Buffer**

Reagent	10ml
Leupeptin	2.5mg/ml
Tris	10 ml (100mM)
EDTA	50µl (1mM)
PMSF	50 µl (1mM)

Buffer of fractions *Leishmania* Soluble Antigens

Buffer A	100mM tris, 1mM EDTA pH 8.0
Buffer B	100mM tris, 1mM EDTA and 1m NaCLpH 8.0

The Values of cells expression

CD	Imm non adherent		Mat non adherent cell		Imm adherent		Mat adherent	
	control	test	control	test	control	test	control	test
CD11c	5.7 %	10.4%	5.44%	17%	16%	13%	0.21%	0.64%
CD205	0.60%	0.09%	0.31%	63.6%	0.49%	0.04%	0.38%	0.14%
CD40	0.60%	0.80%	0.0%	53.5%	0.49%	0%	0.38%	0.04
CD80	0.64%	0.88%	0.06%	65.5%	0.06%	1.86%	11.7%	0.64%
F4/80	0.64%	2.90%	0.06%	0.07%	6%	4.58%	9.85%	7.39%
MHC I	1.20%	35.3%	0.31%	35.3%	1.60%	37%	1.20%	0.33%
MHC II	0.64%	38.6%	0.64%	61.7%	6%	20%	9.85%	48.9%

Table showing perecentage of positive cells for each antibody used

Non-infected DC cell expression %	Infected DC cell expression %
1hr MHC II: control: 1.n44 % test 54.8%	MHC II: control: 0.12% test 8.38%
24hrs MHC II: control: 20% test: 45.5%	24hrs MHC II: control: 0.12% test 2.24%
1hr MHC I: control: 1.78% test: 4.76%	1hr MHC I: control: 1.78% test: 0.93%
24hr sMHC I: control: 8.70% test: 17.6%	24hr MHC I: control: 0.10% test: 0.66%
24hrs CD40 Control: 0.50% test: 4.14	24hr CD40 Control: 0.50% test: 0.08%
24hrs CD80 Control: 0.10 test: 19%	24hrs CD80 Control: 0.36% test: 0.06%
24hrs CD11c Control: 0.12% test 94.8%	24hrs CD11c Control: test: 0.65%
Non-atuclaved DC cell expression %	Atuclaved DC cell expression %
1hr MHC II: control: 0.12 % test 14%	1hrs MHC II: control: 0..33% test 12.5%
24hrs MHC I: control: 20% test: 45.5%	24hr MHC I: control: 1.12% test: 12.7%
1hr MHC II: control: 1.78% test: 4.76%	1hr MHC I: control: 1.17% test: 14%
24hrs MHCII: control: 20% test: 45.5%	24hr sMHC II: control: 0.06% test: 12.7%
24hrs CD40 Control: 0.50% test: 4.14	24hrs CD40 Control: 0.55% test: 14.14
24hrs CD80 Control: 0.10 test: 19%	24hrs CD80 Control: 0.11 test: 20%
24hrs CD11c Control: 0.12% test 94.8%	24hrs CD11c Control: 0.12% test 30%

Table showing perecentage of positive cells for each antibody used

Non-infected DC cell expression %	Infected DC cell expression %	After Treated with fung
MHC I Control: test:33%	MHC I: 0.01%	MHC I: Control: test: 7.40%
MHC II: Control: 0.19 test: 18%	MHC II: Control: 0% test 0%	MHC II: control: 0.06% test 29.7%
MHC I: control: 1.78% test: 4.76%	1hr MHC I: control: 1.78% test: 0.93%	MHC I: control: 1.78% test: 4.14%
MHC I: control: 8.70% test: 17.6%	24hr MHC I: control: 0.10% test: 0.66%	24hr MHC I: control: 1.17% test: 4.14%

Table showing perecentage of positive cells for each antibody used

Animals

Balb/c mice were purchased from the Harlan Olac (Oxon, UK) housed and bred at the Nottingham Trent University. All animals were handled in accordance with the Home Office Codes of Practice for the housing and care of animals.

Phenotyping of the Bone-marrow derived cells

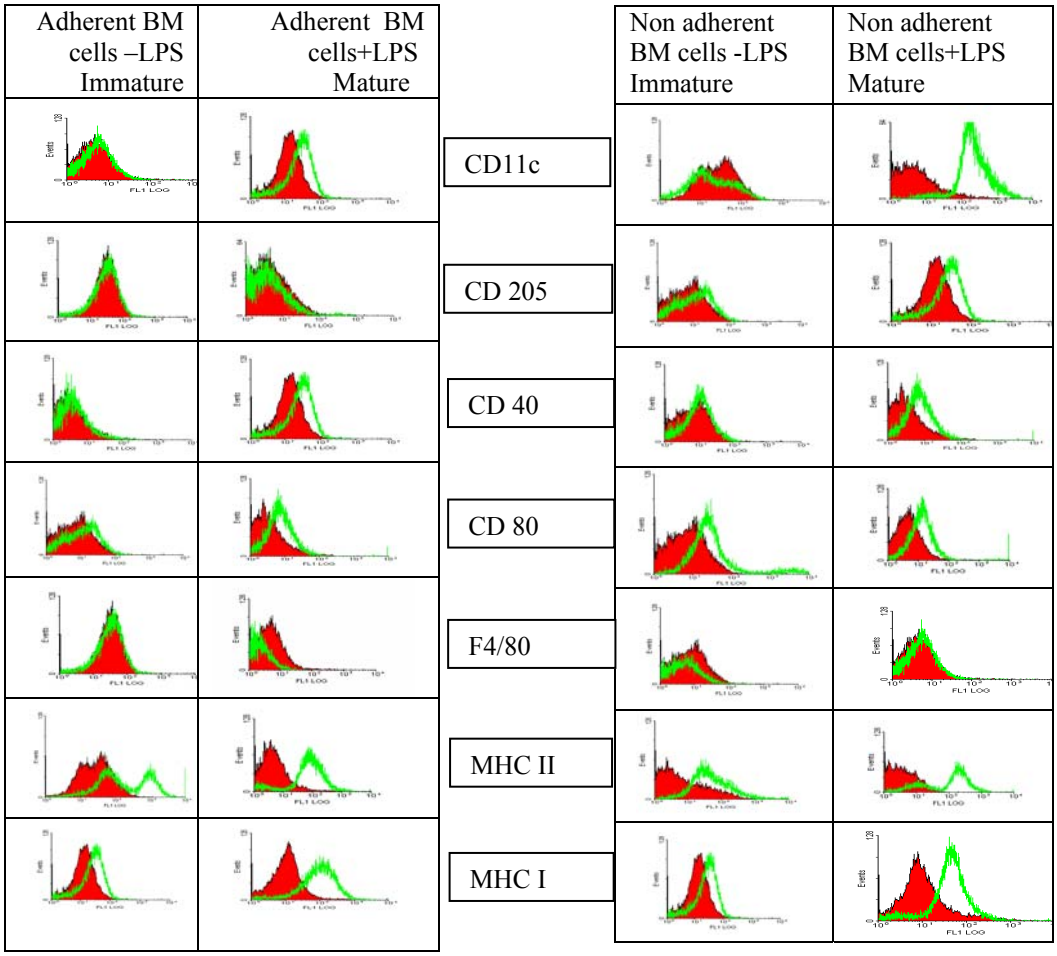
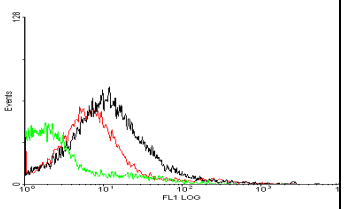
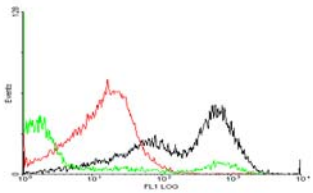
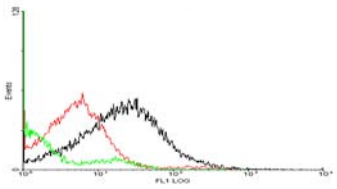
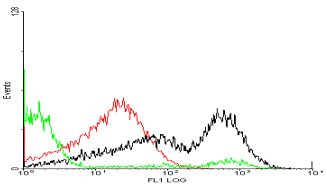
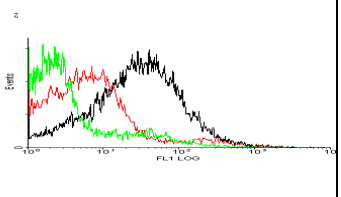
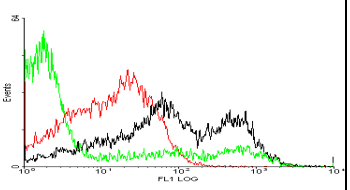
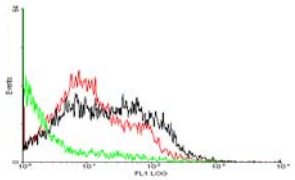
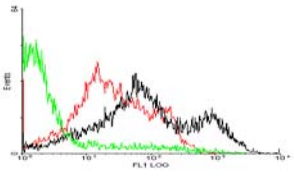


Figure 1: Phenotyping of the Bone-marrow derived cells subgroups using monoclonal antibodies and flow cytometry analysis, red= control, green= test.

The effect of *Leishmania* infection on the expression of MHC class I, MHC class II CD11c, CD80 and CD40

Duration of infection	MHCI	MHCII
1 hour		
3 hour		
5 hour		
24 hour		

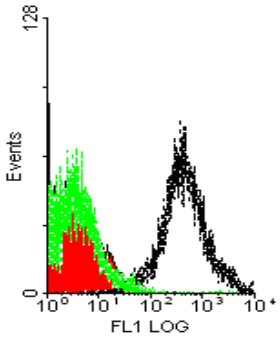
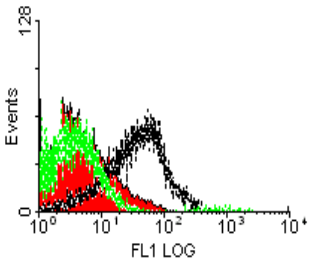
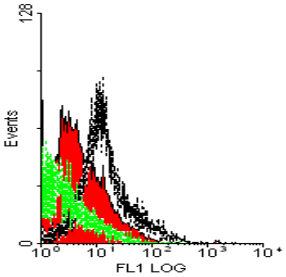
24 hour	24 hour	24 hour
		
CD11c	CD80	CD40

Figure 2: The effect of *Leishmania* infection on the expression of MHC class I, MHC class II CD11c, CD80 and CD40, red= control, green= infected Daces and black= non infected D

Effect of autoclaved parasite on the expression of MHC class I and MHC class II DCs

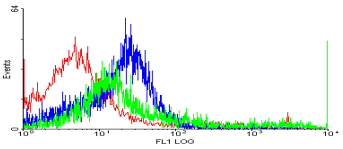
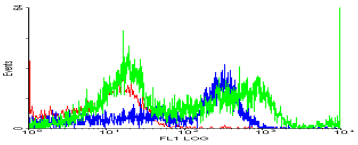
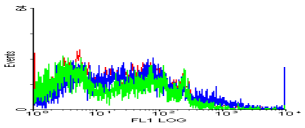
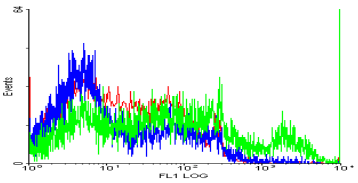
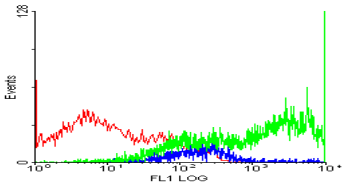
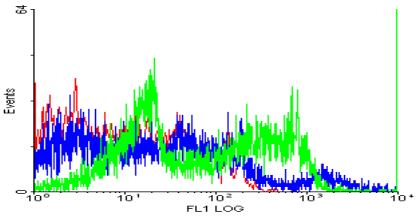
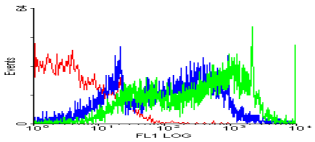
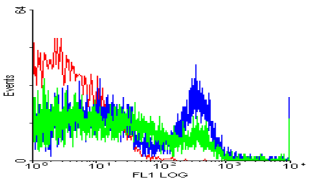
Time course	MHC I	MHC II
1 hour		
3 hour		
5 hour		
24 hour		

Figure 3: Effect of autoclaved parasite on the expression of MHC class I and MHC class II DCs
Red-non infected control (isotype antibody control), blue-non infected test, and green- autoclave infected test.

Effect of fungizone on the expression of MHC I and II in the DCs following *Leishmania* infection

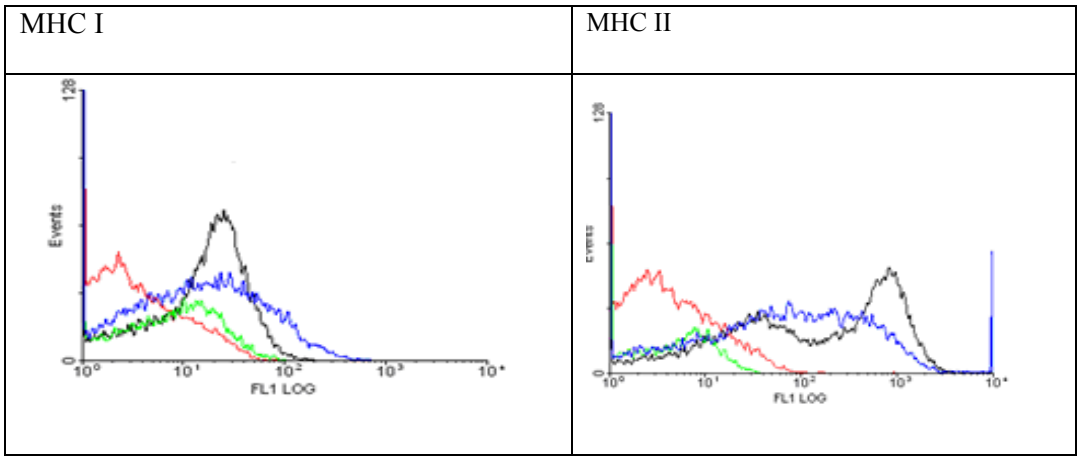
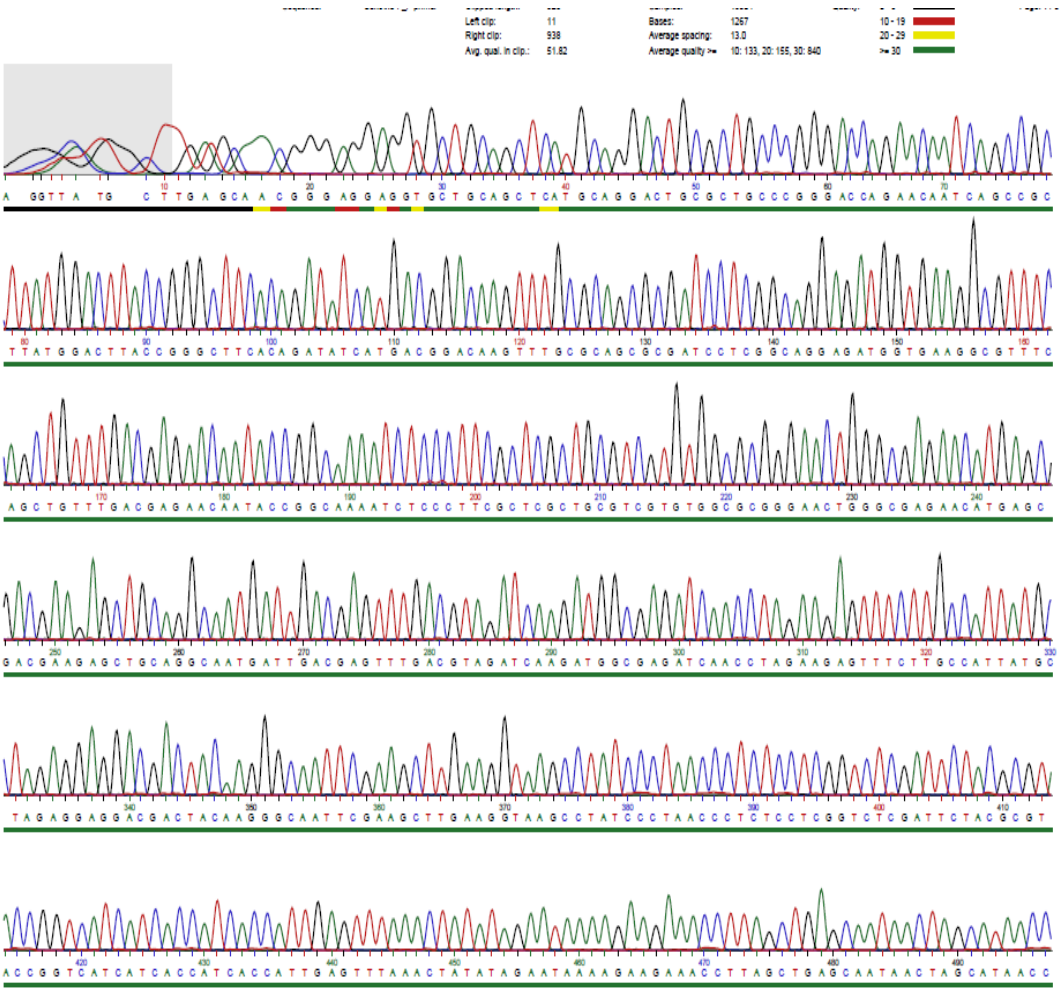


Figure 4: Effect of fungizone on the expression of MHC I and II in the DCs following *Leishmania* infection
Red- non infected control, black-non infected test, green- infected test, blue- infected + fungizone test.

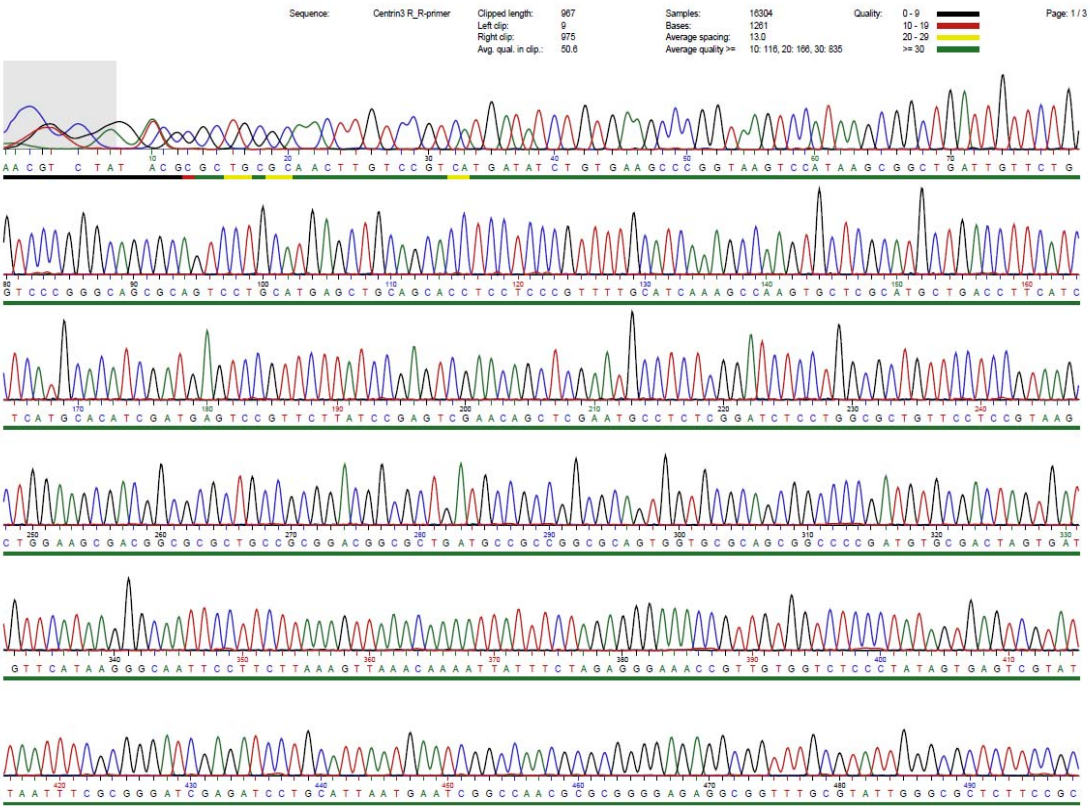
The sequence of *Ldcen-3* gene

ATGAACATCACTAGTCGCACATCGGGGCCGCTGCGCACCACTGCGCCGGC
GGCATCAGCGCCGTCCGCGGCAGCGCGCCGTCGCTTCCAGCTTACGGAGG
CCAACAGCGCCAGGAGATCCG**AGAGGCATTCGAGCTGTTCG**ACTCGGTA
AGAACGGACTCATCGATGTGCATGAGATGAAGGTCAGCATGCGAGCACTT
GGCTTTGATGCAAAACGGGAGGAGGTGCTGCAGCTCATGCAGGACTGCGC
TGCCCGGGACCAGAA**C**AATCAGCCGCTTATGGACTTACCGGGCTTCACAG
ATATCATGACGGACAAGTTTGCGCAGCGCGATCCTCGGCAGGAGATGGTG
AAGGCGTTTCAGCTGTTTGACGAGAACAATACCGGCAAAATCTCCCTTCG
CTCGCTGCGTCGTGTGGCGCGGGAACTGGGCGAGAACATGAGCGACGAA
GAGCTGCAGGCAATGATTGACGAGTTTGACGTAGA**TCAAGATGGCGAGA**
TCAACCTAGAAGAGTTTCTTGCCATTATGCTAGAGGAGGACGACTAC

Ldecn-3 forward primer



***Ldecn-3* Reverse primer**

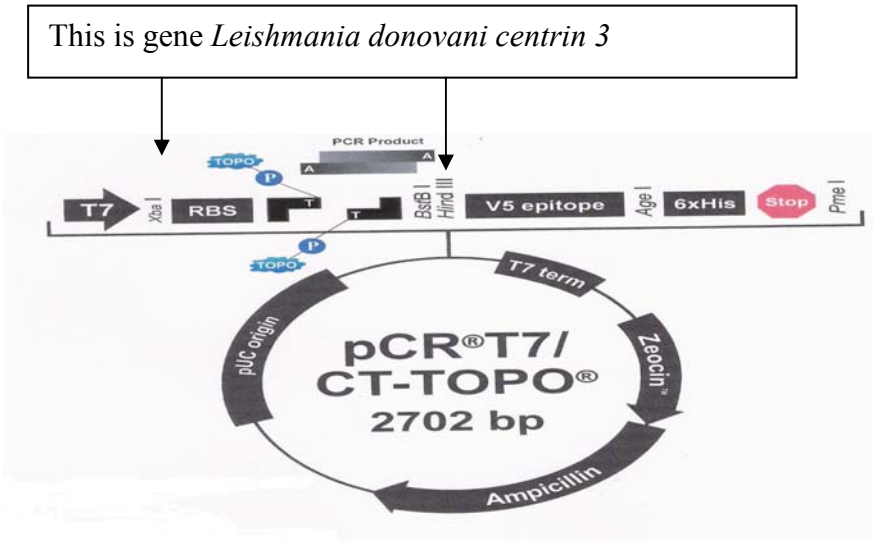


The recognition sites of these enzymes are given below

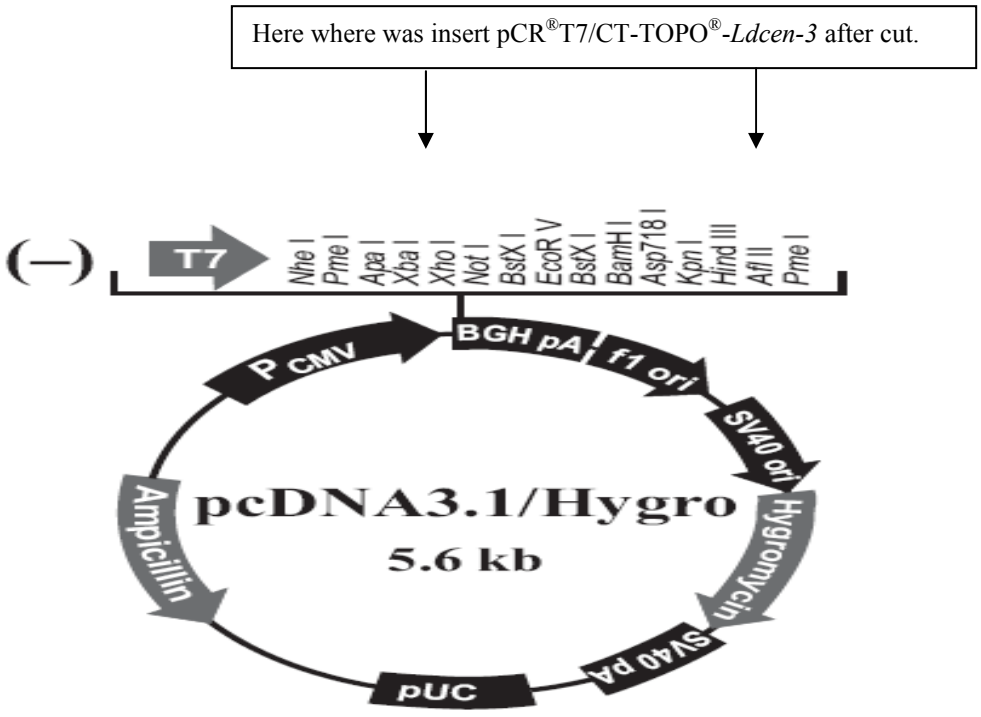
Hind111:- 5’ A AGCT T 3’
3’ T TCGA A 5’

Xba1:- 5’ T CTAG A 3’
3’ A GATC T 5’

The recognition site for these two enzymes were not present in the *Ldcen-3* gene sequence, so these two enzymes were conveniently used for restriction digestion purpose



A -Cutting pCR®T7/CT-TOPO®-*Ldcen-3* by XbaI and HinIII, then subcloned to pcDNA3.1 (-)



Cutting PCDNA 3.1(-) by XbaI and HinIII A

Chapter 8 References

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